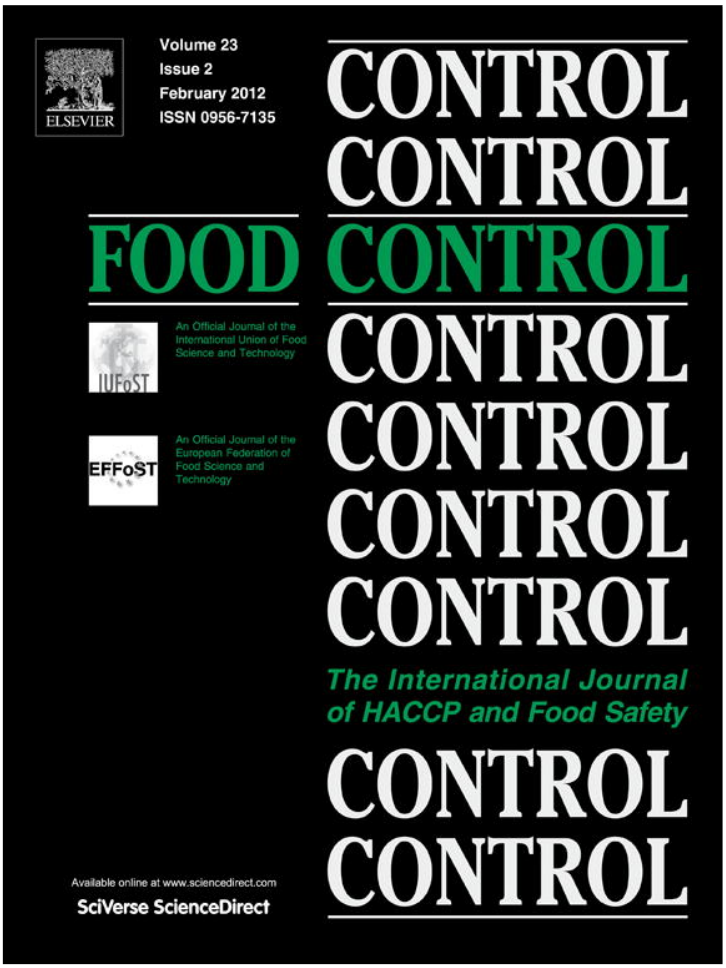

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Enteropathogenic (EPEC) and Shigatoxigenic *Escherichia coli* (STEC) in broiler chickens and derived products at different retail stores

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ABSTRACT

Enteropathogenic (EPEC) and Shigatoxigenic *Escherichia coli* (STEC) are foodborne pathogens that cause potentially fatal infant diarrhea and hemolytic uremic syndrome, respectively. We investigated the presence of intimin and Shiga toxin encoding genes, as indicators of EPEC and STEC presence in cloacae and chicken products. The analyzed products were hamburgers, giblets and carcasses obtained from poultry and butcher shops. EPEC contamination predominated over STEC contamination in cloacae and chicken products, although some differences were detected when the kind of food or shop was taken into account. In particular, among chicken hamburgers we found a greater proportion of EPEC than STEC-positive samples at poultry shops, while in butcheries STEC was predominant. This finding could suggest cross contamination during handling at butcheries. The results indicate that it is necessary to improve hygienic measures both during slaughtering and manipulation of chicken products at retail stores, to provide a safe product to consumers.

1. Introduction

Attaching and effacing *Escherichia coli* (AEEC) comprise enteropathogenic *E. coli* (EPEC) and several Shiga toxin-producing *E. coli* (STEC) strains. AEEC are characterized by their ability to cause attaching and effacing (A/E) intestinal lesions, mediated by intimin (encoded by the chromosomal gene *eae*), among other proteins (Knutton, Baldwin, Williams, & McNeish, 1989). EPEC can also harbor a type IV pilus (BFP) with a major structural subunit encoded by *bfpA* gene present in EPEC adherence plasmid (EAF). Depending on the presence or absence of these pili, these strains are classified as typical and atypical, respectively.

The main virulence factors of STEC are Shiga toxins (Stx) 1 and 2 encoded by *stx* genes, which inhibit protein synthesis in mammalian cells and are absent in EPEC strains (Donnenberg et al., 1993; Nataro & Kaper, 1998). Additional virulence-associated markers, among many others, are a plasmid-encoded enterohemolysin (Schmidt, Beutin, & Karch, 1995) and, in strains lacking *eae*, an autoagglutinating adhesin (Saa) which could be involved in adhesion (Paton, Srimanote, Woodrow, & Paton, 2001).

EPEC and STEC are important pathogens related to Public Health. EPEC produce potentially fatal infant diarrhea, noticeably in developing countries (Nataro & Kaper, 1998; Trabulsi, Keller, & Gomes, 2002), and have been isolated from different animal species (Blanco et al., 2005; Cortés et al., 2005; Krause, Zimmermann & Beutin, 2005; Nataro & Kaper, 1998) and from a variety of foods, including chicken products (Nataro & Kaper, 1998; Omaye, 2004).

STEC cause Hemorrhagic Colitis (HC) and Hemolytic Uremic Syndrome (HUS) in humans (Karmali, 1989; Tarr, Gordon, & Chandler, 2005). The predominant serotype isolated from HUS cases is O157:H7, but non-O157:H7 STEC are frequently involved and their importance is increasing worldwide (Bettelheim, 2007).

Ruminants, especially cattle, are the main reservoir of STEC in different countries (Blanco et al., 2004; Caprioli, Morabito, Brugreb, & Oswald, 2005; Fernández, Rodríguez, Arroyo, Padola, & Parma, 2009; Meichtri et al., 2004; Padola et al., 2004; Parma et al., 2000) and the transmission to humans occurs mainly by contaminated foods of animal origin, as well as cross contamination due to inadequate food manipulation (Gyles, 2007). On some occasions

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the consumption of chicken products has been related to cases of HUS (Rivas, 2009; Vernozy-Rozand & Ray-Gueniot, 1997) but most of the studies have been focused on the detection of O157:H7 in these products (Abdul-Raouf, Ammar, & Beuchat, 1996; Akkaya, Atabay, Kenar, & Alisarli, 2006; Chinen et al., 2009; Doyle & Schoeni, 1987; Reuben, Treminio, Arias, & Chaves, 2003; Vernozy-Rozand & Ray-Gueniot, 1997).

The occurrence of EPEC and STEC contamination in chicken meat can be related to the evisceration process, mainly to the rupture of the animal intestine, or possible cross contamination during food processing and handling, which can differ among retail shops. At butcheries chicken meat is often stored and exposed in the same refrigerator as bovine meat and, in addition, the grinding-machine used to prepare chicken hamburgers is shared with bovine meat. Contrarily, poultry shops mainly sell poultry products. In both types of shops, the hamburgers are made with chicken breast meat, chicken skin, salt, condiments, and, only in butcheries, bovine fat is added to the mix. Giblets containing liver, heart, stomach and neck are packed in individual bags inside the chicken carcasses.

Although there is some information about the presence of STEC and EPEC in chickens, there are no studies that detect these two pathotypes simultaneously in retail markets. Besides, there is scarce information about non-O157 STEC and EPEC in different chicken products. Therefore, the aim of this study was to detect Shiga toxin and intimin encoding genes in cloacae of chickens from different farms, and in chicken products (hamburgers, giblets and carcasses), collected from butcheries and poultry shops, to infer and compare STEC and EPEC contamination.

2. Materials and methods

2.1. Sample collection

Chicken samples were obtained from 4 butcheries and 5 poultry shops from one city in Argentina. Each shop was sampled at least twice in the period from April 2007 to December 2009. At butcheries 140 chicken hamburgers, 106 giblets and 160 carcasses were collected, and at poultry shops, 160 chicken hamburgers, 194 giblets and 297 carcasses. Carcasses were sampled with 2 swabs, one across the external surface and the other across the visceral cavity surface. In addition, cloacal swabs were collected from 559 chickens from 3 small-scale intensive management broiler farms and from 300 chickens at a broiler-producing company. Swabs were placed in Stuart transport medium and processed immediately at the laboratory.

2.2. Sample preparation

Ten grams of hamburger or giblet samples were cultured in 100 ml of buffered peptone water with shaking (100 rpm) at 37 °C for 18 h. An aliquot (1750 μl) was centrifuged 4 min at 500×*g*, and then 1300 μl of supernatant was centrifuged 3 min at 13,000×*g*. The pellet was resuspended in 34 μl of sterile double-distilled water. It was boiled for 10 min; centrifuged 2 min at 13,000×*g* and the crude supernatant was used for PCR (modified from Uyttendaele et al., 1998).

Swabs were processed according to Etcheverría et al. (2010) and Fernández et al. (2009), with modifications. Briefly, the swabs were cultured in buffered peptone water (carcass swabs) and Luria Bertani broth (cloacal swabs) with shaking (100 rpm) at 37 °C for 18 h, and an aliquot was boiled after diluting it in sterile double-distilled water.

Table 1
Chicken hamburger contamination at different shops.

	Shops		Total ($n = 300$)
	Poultry ($n = 160$)	Butcheries ($n = 140$)	
EPEC +	49 (30.6%)	14 (10.0%)	63 (21%)
STEC +	2 (1.2%)	29 (20.7%)	31 (10.3%)
STEC/EPEC +	6 (3.7%)	17 (12.1%)	23 (7.7%)

2.3. Detection of stx_1, stx_2 and *eae* genes

In this study, samples *eae*-positive but *stx*-negative are considered as contaminated with EPEC, and samples *stx*-positive but *eae*-negative as contaminated with STEC. Those presenting both *stx* and *eae* could be contaminated with STEC and EPEC or with STEC harboring *eae* (this kind of contamination is referred in this manuscript as STEC/EPEC).

Multiplex PCR was used for the detection of stx_1, stx_2 and *eae* genes with primers and experimental conditions described by Paton and Paton (2002). PCR-amplified products were electrophoresed in 2% agarose gels and stained with ethidium bromide. *E. coli* O157:H7 strain EDL933 (kindly supplied by Dr. J. Blanco, Reference Laboratory for *Escherichia coli*, Lugo, Spain) was used as positive control and double-distilled water as negative control.

2.4. Detection of *eaeγ1* gene

Each culture positive for *stx* was tested for the presence of *eaeγ1* gene, characteristic of O157 serogroup (Gannon, Rashed, King, & Golsteyn Thomas, 1993; Oswald et al., 2000), according to the strategy of Padola et al. (2004). *E. coli* O157:H7 strain EDL933 was used as positive control, and double-distilled water as negative control.

2.5. Detection of *bfpA*

All *eae*-positive samples were tested for the presence of *bfpA* by monoplex PCR using the method of Gunzburg, Tornieporth, and Riley (1995). *E. coli* O157:H45 ($bfpA^+$) (kindly supplied by Dr. A. Bentancor, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Argentina) was used as positive control, and double-distilled water as negative control.

2.6. Statistical analysis

Fisher's exact test was used to determine the association between genes detected in hamburgers and giblets and their origin (poultry shops or butcheries). This analysis was made by using the procedure "Proc Freq" (SAS 9.2, Cary, NC).

Multinomial data analysis was used to evaluate the distribution of Shiga toxins genes (stx_2, stx_2-stx_1 and stx_1) among different types of shops and chicken products. This analysis was made by using the "Proc Genmod" (SAS 9.2).

Table 2
Chicken giblets contamination at different shops.

	Shops		Total ($n = 300$)
	Poultry ($n = 194$)	Butcheries ($n = 106$)	
EPEC +	82 (42.3%)	48 (45.3%)	130 (43.3%)
STEC +	2 (1.0%)	5 (4.7%)	7 (2.3%)
STEC/EPEC +	2 (1.0%)	1 (0.9%)	3 (1%)

Table 3
Chicken carcass contamination with regard to the surface sampled.

	E[a]	V[b]	E and V[c]
EPEC (18/457)	3	13	2
STEC (15/457)	7	5	3

[a] E: number of carcasses positive only at the external surface.
[b] V: number of carcasses positive only at the visceral cavity surface.
[c] E and V: number of carcasses positive both at the external and visceral cavity surfaces.

3. Results

Among the 1057 chicken products, samples positive for *stx*$_1$, *stx*$_2$ and/or *eae* were detected at different shops, suggesting contamination with EPEC (20%), STEC (5%) or both (2.4%). Regarding cloacal samples, EPEC-positive samples also predominated over STEC. All types of samples were negative for *eae*γ*1* and *bfpA*. The results are shown below, grouped according to the type of sample.

3.1. Chicken hamburgers

Of the total of chicken hamburger samples, 21% were contaminated with EPEC, 10.3% with STEC and 7.7% with STEC/EPEC (Table 1).

At poultry shops we found a greater proportion of EPEC-positive samples (30.6%) but in contrast, at butcheries STEC-positive samples were predominant (20.7%), and these differences were significant ($p < 0.0001$).

3.2. Giblets

Among giblets samples, 43.3% were contaminated with EPEC, 2.3% with STEC and 1% with STEC/EPEC (Table 2). Both at poultry shops and butcheries, we found a greater proportion of EPEC- than STEC-positive samples. Neither the percentage of *eae*-positive samples differed between the two kinds of shops ($p = 0.6292$), nor the percentage of *stx*-positive samples ($p = 0.1743$).

3.3. Carcasses

A low number of carcasses were contaminated with STEC (3.3%) and EPEC (3.9%), in a similar proportion at both kinds of shops. Although these bacteria were present on external or visceral cavity surfaces or both, the following tendency was observed ($p = 0.0804$). Regarding EPEC, a greater proportion of samples from the visceral cavity surface were positive than at the external surface, but for STEC a similar proportion of positive samples were found at each surface (Table 3).

3.4. Cloacae

Of 859 cloacal samples, 102 (11.9%) were contaminated with EPEC and, in contrast, only one sample (0.1%) was contaminated with STEC.

3.5. Distribution of stx genes among STEC-positive chicken products at different shops

The distribution of *stx* genes among some types of samples significantly differed between poultry shops and butcheries ($p = 0.0222$). At poultry shops, we observed that *stx*$_2$ predominated over both *stx*$_1$ and *stx*$_1$/*stx*$_2$ among chicken hamburgers, giblets and carcasses. The same trend was observed in hamburgers and giblets from butcheries ($p = 0.2661$), but in carcasses *stx*$_1$ was predominant ($p = 0.0003$) (Table 4). It is important to note that all the *stx*$_1$–positive carcass samples were detected in one sampling occasion at the same shop and therefore it would not indicate a trend.

4. Discussion

The present study demonstrates the presence of Shiga toxin and intimin encoding genes in cloacae and retail chicken products in Argentina. We looked for the presence of *stx* and *eae* as indicator of sample contamination with STEC or EPEC, respectively. Nowadays, the detection of these genes without the corresponding strain isolation is considered a presumptive diagnosis but it is valid for the identification of carriage status (Etcheverría et al., 2010; Fernández et al., 2009).

In chicken products, EPEC contamination predominated over STEC contamination, although some differences were detected when the kind of food or shop was taken into account. Considering chicken hamburgers, EPEC contamination was found in a greater proportion than STEC only at poultry shops. As far as we know, there is no published information about the presence of EPEC in this kind of sample, while there are some reports of this pathotype in chicken meat samples (Kobayashi, Pohjanvirta, & Pelkonen, 2002; Lee, Jang, Hwang, & Rhee, 2009).

A greater proportion of chicken hamburgers contaminated with STEC were detected more frequently at butcheries than at poultry shops, suggesting that the presence of STEC could be indicating cross contamination during handling at butcheries, probably from other sources like bovine meat. Moreover, Etcheverría et al. (2010) found that the proportion of STEC-contaminated bovine meat increased from slaughters to butcheries, augmenting the risk of cross contamination during manipulation at butcheries and Chinen et al. (2009) isolated identical O157:H7 strains from uncooked and cooked beef and chicken hamburgers.

In giblets samples, EPEC contamination was found in a high proportion (more than 40%) at both kinds of shops, whereas STEC were found in a low proportion (less than 10%). The manipulation of the viscera and the necks after their remotion may increase the risk of giblets contamination. Reuben et al. (2003) reported the isolation of STEC O157:H7 (3%) in this kind of samples in Costa Rica but we did not find studies regarding the presence of non-O157 or EPEC in giblets.

In our study, EPEC were present in 3.9% of chicken carcasses, finding a greater proportion of positive samples from visceral cavity

Table 4
Distribution of *stx* genes among STEC-positive samples from butcheries (B) and poultry shops (P).

	No. of positive samples						
	Hamburgers		Giblets		Carcasses		Total
	B	P	B	P	B	P	
stx$_2$	27	5	5	2	1	4	44
stx$_2$/*stx*$_1$	15	3	1	1	1	0	21
stx$_1$	4	0	0	1	7	2	14
Total	46	8	6	4	9	6	79

surfaces than from external surfaces. This finding can be related with the higher probability of contamination of this surface during the evisceration process and possibly with the difficulty of removing it with the washing steps. Lillard (1991) detected significantly higher levels of *Enterobacteriaceae* in the visceral cavity from pre- and post-chill broilers than in the external surface, indicating a more effective washing of the surface than the cavity of the bird. Another aspect that could contribute to internal contamination is that ready to sell carcasses contain a plastic bag with the giblets inside the visceral cavity. As far as we know, there are no reports about the presence of EPEC at the visceral cavity surface, instead other authors sampled only the external surface and detected this pathotype in around 10% of carcasses (González, Rosa, Andrade, & Tibana, 2000; Nzouankeu, Ngandjio, Ejenguele, Njine, & Ndayo Wouafo, 2010).

Regarding STEC, 3.3% of chicken carcasses were contaminated, with a similar proportion of positive samples from either surface. Akkaya et al. (2006) and Chinen et al. (2009), detected 1 and 8% (respectively) of chicken carcasses contaminated with STEC O157:H7, but these researchers used selective methods for the isolation and only sampled the external surface.

We found higher EPEC than STEC contamination in chicken cloacal samples (12% vs. 0.1%). Kobayashi, Kanazaki, Hata, and Kubo (2009) and Kobayashi et al. (2002) detected intimin-producing *E. coli* in cloacal swabs from 25% of wild birds and 15% of broilers in Japan. Other authors found EPEC in chicken faecal samples (Farooq, Hussain, Bhat, & Wani, 2009; Krause, Zimmermann, & Beutin, 2005; Wani, Samanta, Bhat, & Nishikawa, 2004) and Kariuki et al. (2002) demonstrated that apparently healthy chickens may carry EPEC strains, suggesting that chicken could be a reservoir of these bacteria.

In our study, we found only one (0.1%) cloacal sample positive for STEC. As far as we know, there are no studies about the presence of non-O157:H7 STEC in cloacal samples, instead, Dipineto et al. (2006) detected STEC O157:H7, with a low prevalence (4%), in cloacal samples of layer hens.

None of the chicken samples were positive for *eae*$\gamma 1$, suggesting that STEC O157:H7 was not present in the samples analyzed. Similarly, other researchers could not found this serotype in cloacal samples, raw chicken meat and carcasses, and ready to eat chicken, although they used a selective methodology (Balagué et al., 2005; Blanco, Blanco, Mora, & Blanco, 1997; Chapman, Siddons, Cerdan Malo, & Harkin, 1997; Read, Gyles, Clarke, Lior, & McEwen, 1990; Tutenel, Pierard, Van Hoof, Cornelis, & De Zutter, 2003).

The *bfpA* gene was not detected in any of the samples we analyzed, indicating that *eae*-positive chicken samples were contaminated with atypical EPEC, in concordance with the findings of Farooq et al. (2009); Kobayashi et al. (2002) and Krause, Zimmermann and Beutin (2005).

In this study, we found that stx_2 was the predominant gene over stx_1 and stx_1–stx_2 in STEC-positive samples from chicken products, and the same trend has been observed in cattle and bovine meat in our country (Blanco et al., 2004; Chinen et al., 2003; Etcheverría et al., 2010; Fernández et al., 2009; Padola et al., 2004). This is important because stx_2-producing strains are more related with HUS than stx_1-producing strains (Paton & Paton, 2002).

To our knowledge, this study is the first that reports and compares STEC and EPEC contamination in chicken and their products analyzing the differences among products and their origin (butcheries or poultry shops).

5. Conclusion

The results showed EPEC and STEC contamination in chicken products, with some differences between different types of shops. In particular, STEC contamination could result from cross contamination during handling, especially at butcheries, while EPEC contamination may result from contamination at slaughter, as EPEC were detected in cloacal samples of live chickens. Therefore, it is necessary to improve hygienic measures both during slaughtering and manipulation of chicken products at retail stores, to provide a safe product to consumers.

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