MICROBIOLOGY AND FOOD SAFETY

Presence of pathogenicity islands and virulence genes of extraintestinal pathogenic *Escherichia coli* (ExPEC) in isolates from avian organic fertilizer

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ABSTRACT Poultry litter is commonly used as fertilizer in agriculture. However, this poultry litter must be processed prior to use, since poultry have a large number of pathogenic microorganisms. The aims of this study were to isolate and genotypically and phenotypically characterize *Escherichia coli* from avian organic fertilizer. Sixty-four *E. coli* isolates were identified from avian organic fertilizer and characterized for ExPEC virulence factors, pathogenicity islands, phylogenetic groups, antimicrobial resistance, biofilm formation, and adhesion to HEp-2 cells. Sixty-three isolates (98.4%) showed at least one virulence gene (*fimH*, *ecp*A, *sit*A, *tra*T, *iut*A, *iro*N, *hly*F, *omp*T and *iss*). The predominant phylogenetic groups were groups A (59.3%) and B1 (34.3%). The pathogenicity island CFT073II (51.5%) was the most prevalent among the isolates tested. Thirty-two isolates (50%) were resistant to at least one antimicrobial agent. Approximately 90% of isolates adhered to HEp-2 cells, and the predominant pattern was aggregative adherence (74.1%). In the biofilm assay, it was observed that 75% of isolates did not produce biofilm. These results lead us to conclude that some *E. coli* isolates from avian organic fertilizer could be pathogenic for humans.

Key words: *Escherichia coli*, organic fertilizer, virulence factors, antimicrobial resistance, poultry

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INTRODUCTION

In 2011, Brazilian chicken meat production reached 13.05 million tons, maintaining its position as the third-largest producer after the USA and China and the first in global exportation (UBABEF, 2014). Consequently, with increases in poultry production, high amounts of poultry litter waste are produced. Approximately 1.75 kg of poultry litter are produced per kilogram of poultry (Santos et al., 2005); thus, in 2011, Brazil produced approximately 22.83 million tons of poultry litter.

Manure, or animal waste, is commonly used as fertilizer in organic and conventional agriculture mainly for vegetable production (Johannessen et al., 2004; Oliveira et al., 2010). The main fertilizer used is poultry litter; however, the use of this poultry litter can lead to problems, such as pollution by nutrients, chemicals, and microorganisms (Hahn, 2004), and the microorganisms can survive for months (Merchant et al., 2012). Thus, this waste must be properly treated before being used. Some farmers use poultry litter following the Normative Instruction n° 007, Ministry of Agriculture and Supply, which allows the use of animal waste since these compounds are free of contaminants (BRASIL, 1999); however, the normative document did not specify how this material should be processed.

The composting process is a widely used method to make organic waste safe prior to use (Wilkinson et al., 2011). During the initial stages, called the bio-oxidation phase, simple organic compounds are metabolized by the microorganisms, resulting in CO_2, NH_3, water vapor, and heat, which accumulate in the windrow, raising the temperature. High temperatures reduce the number of pathogens and weeds from an organic compound, further decreasing its volume (Bernal et al., 2009).

Organic fertilizer presents a viable alternative to other production systems, with benefits such as large amounts of nutrients, low levels of pathogens (bacteria, parasites, and weeds), easy handling and stocking, and low production costs (Sweeten and Auvermann, 2008).

Among the diverse bacteria present in organic fertilizer, there are some gram-positive and gram-negative

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organisms (Zhong et al., 2010). *Escherichia coli* is a gram-negative bacteria that belongs to the Enterobacteriaceae family, and it is present in the intestinal tract of humans and other animals. *E. coli* strains are categorized into commensal, diarrheagenic *E. coli* (DEC) and extraintestinal pathogenic *E. coli* (ExPEC) based on virulence profile and clinical reports (Kaper et al., 2004).

DEC strains were already found in avian organic fertilizers (Puño-Sarmiento et al., 2014). Although *E. coli* is known to cause intestinal diseases, the frequency, diversity, potential severity, and economic impacts of infections caused by ExPEC also have great importance (Mellata et al., 2003). ExPEC strains harbor virulence genes that allow them to cause extraintestinal infections. The virulence characteristics present in most ExPEC strains include iron acquisition mechanisms (siderophores), fimbrial adhesins such as type 1 and P, serum resistance, capsule, and toxins such as hemolysin and cytotoxic necrotizing factor type 1 and 2 (Russo et al., 2002).

Furthermore, the increase in the prevalence of antimicrobial resistance in microbial communities is worrying (Miller et al., 2013). In clinical veterinary practice, antibiotics are used for therapy, prevention of bacterial infections and as growth promoters, increasing the efficacy of feed (Furtula et al., 2010). Also, studies have shown that antibiotic resistance genes can be transferred between commensal and pathogenic bacteria through mobile plasmids in poultry (Poppe et al., 2005; Poole et al., 2006), and between strains of animal compost and manure (Guan et al., 2007).

It is important to characterize the bacteria present in fertilizer of avian origin, since these bacteria may spread resistance genes and also be pathogenic for humans. Therefore, our study aimed to detect the presence of genes encoding ExPEC virulence factors, antimicrobial resistance, and other phenotypic characteristics in *E. coli* isolates from avian organic fertilizer.

MATERIALS AND METHODS

Organic Fertilizer Samples and Bacterial Strains

From December 2011 to June 2012, 40 samples of avian organic fertilizers were collected from 12 farms in the Londrina region (Northern Paraná, Brazil). The organic fertilizer used in this study was a final product of the poultry litter composting process. Approximately 25 g of organic fertilizer was collected using latex gloves and transported to the laboratory into sterilized glass jars that were stored in a refrigerator (4°C). The samples were diluted on physiological saline sterile solution and plated on MacConkey agar (Oxoid®, Basingstoke, Hants, UK) and incubated at 37°C for 24 h. Two to 3 *E. coli* by colony morphology were selected and submitted to biochemical assays such as EPM, MILi and Simmons Citrate agar (Toledo et al., 1982a, 1982b; Ewing, 1986). The confirmed isolates were stored in Brain Heart Infusion (BHI) (Difco®, Sparks, MD) plus 25% glycerol (Sigma®, St. Louis, MO) media at −20°C and −80°C. Negative and positive controls used in this study belong to the bacterial collection of the Laboratory of Basic and Applied Bacteriology, Department of Microbiology, Universidade Estadual de Londrina.

DNA Extraction

The strains were grown in Luria Bertani broth (LB) (Difco®, Sparks, MD) at 37°C for 24 h. After centrifugation, pellets from 1 mL of growth culture were resuspended in 200 μL of sterile water, boiled for 10 min and centrifuged at 12,000 × *g* for 6 min. The supernatant were used as DNA template for the PCR assays.

Virulence Genotyping

All isolates were examined for the presence of 5 genes that are predictors of the virulence of APEC (*iut*A, *iro*N, *hly*F, *iss* and *omp*T), using pentaplex-PCR as described by Johnson and collaborators (2008) (Table A1). Twelve other virulence genes found in ExPEC strains were used to characterize the bacterial isolates': 1) bacterial adhesion (*pap*C, *papG, tsh, ecp*A and *fim*H), 2) iron acquisition (*fyu*A and *sit*A), 3) invasion (*ibe*A), 4) serum resistance (*tra*T), and 5) toxin genes (*hly*A, *cnf-1* and *cnf-2*). All genes were detected by PCR assays as previously described (Table A1).

Determination of Phylogenetic Groups

E. coli strains were classified by phylogenetic group using a PCR technique previously described by Clermont and collaborators (2000). The genes *chu*A (required for heme transport in *E. coli* O157:H7), *yja*A (initially identified in the recent complete genome sequence of *E. coli* K-12), and a DNA fragment, *TspE4C2* (anonymous fragment), were included in this assay (Table A1).

Pathogenicity Islands (PAI)

The presence of the main PAIs was carried out using 2 multiplex-PCR reactions, as previously described by Sabaté and collaborators (2006). For the first multiplex-PCR, the PAIs identified were: PAI III536 (S-fimbriae and an iron siderophore system); PAI IV536 (yersiniabactin siderophore system); PAI IICFT073 (P-fimbriae and iron-regulated genes) (Table A1); and for the second multiplex-PCR the PAIs identified were: PAI I536 (α-hemolysin, CS12 fimbriae and F17-like fimbrial adhesin); PAI II536 (α-hemolysin and P-related fimbriae); PAI IJ96 (α-hemolysin and P-fimbriae); and PAI ICFT073 (α-hemolysin, P-fimbriae and aerobactin) (Table A1).

Adherence and Biofilm Tests

HEp-2 cells were used in adherence assays as previously described by Cravioto and collaborators (1979), with slight modifications. The HEp-2 cells were grown in 24-well sterile microplates (BD Falcon, Bedford, MA) and round cover slips (13 mm in diameter) were placed prior to inoculation. The cells were grown in each well with 1 mL of Dulbecco's Modified Eagle Medium (DMEM, Invitrogen®, Carlsbad, CA) containing 10% fetal calf serum (Invitrogen®, Carlsbad, CA) for 24 h at 37°C with 5% CO_2. The cells were used at 70% confluence. The plates were washed twice with 0.01 M phosphate-buffered saline (PBS), pH 7.4. One milliliter of DMEM supplemented with 2% fetal calf serum and 3% D-mannose (Sigma®, St. Louis, MO) and 40 μL of bacterial culture incubated overnight in LB broth were added into each well and incubated at 37°C for 3 h. After the cells had grown, the wells were washed twice with PBS, DMEM containing 2% fetal calf serum was added, and the cells were incubated for 3 h. Next, the wells were washed once with PBS, fixed with absolute methanol (Merck®, Darmstadt, DEU) for 15 min and stained with May-Grunwald (Sigma®, St. Louis, MO) and Giemsa (Sigma®, St. Louis, MO) for 10 min each. The slides were observed using a light microscope with oil immersion lens. The patterns of adhesion were determined as previously described by (Nataro et al., 1987; Scaletsky et al., 2005).

Biofilm assays were performed according to methodology previously described by Wakimoto and collaborators (2004) with slight modifications. All *E. coli* strains were grown in LB broth for 24 h at 37°C. Five microliters of culture was inoculated in 195 μL of DMEM containing 0.45% glucose in 96-well polystyrene plates (BD Falcon, Bedford, MA), and incubated for 24 h at 37°C. The microplates were washed twice with 0.01 M PBS, pH 7.4. Each sample was stained for 5 minutes with 0.5% crystal violet and washed 4 times with 0.01M PBS, pH 7.4, and 200 μL of 95% (v/v) ethanol was added. Quantification was carried at 570 nm using an automated plate reader (Synergy^{TM} HT, Biotek, Winooski, VT). The biofilm formation data were expressed as means ± SD. Based on the results from the biofilm assay, the strains were classified into 3 groups according to absorbance: no biofilm production ($OD_{570nm} < 0.1$), weak biofilm production ($0.1 \leq OD_{570nm} \geq 0.2$), and strong biofilm production ($OD_{570nm} > 0.2$).

The adherence and biofilm assays reference strain EAEC 042 (*agg*R+, aggregative adherence) was used as a positive control, and *E. coli* K12 HB101 was used as a negative control.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was determined using the disk-diffusion method, by referencing the guidelines set forth by the Clinical and Laboratory Standards Institute (CLSI, 2008; CLSI, 2012). All *E. coli* isolates were tested for antimicrobial agents after previous inoculation on Mueller Hinton agar (Difco®, Sparks, MD) using a swab. The following antimicrobial agents were used: amoxicillin (AMO, 10 μg), amoxicillin-clavulanic acid (AMC, 30 μg), ampicillin (AMP, 10 μg), aztreonam (ATM, 30 μg), cefotaxime (CTX, 30 μg), chloramphenicol (CHL, 30 μg), ciprofloxacin (CIP, 5 μg), colistin (COL, 10 μg), gentamicin (GEN, 10 μg), imipenem (IPM, 10 μg), nalidixic acid (NAL, 30 μg), norfloxacin (NOR, 10 μg), polymyxin B (POL, 300U), tetracycline (TET, 30 μg), trimethoprim-sulfamethoxazole (SXT, 25 μg), and streptomycin (STR, 10 μg) (Laborclin®, Pinhais, Paraná, Brazil). Enrofloxacin (ENR, 5 μg) (Laborclin®, Pinhais, Paraná, Brazil) was also tested because this antimicrobial is commonly used in veterinary clinics. *E. coli* strain ATCC 25922 was used as a quality control for antimicrobial susceptibility testing.

Statistical Analysis

Statistical analyses were performed using the X^2 (Chi-square) test for the bacterial groups. Differences were considered significant at $P < 0.05$, and the data were expressed as the means ± SD. The statistical analyses were performed using the *BioEstat* version 5.3 software.

RESULTS

E. coli Isolates, Virulence Genes and Phylogenetic Groups

A total of 64 *E. coli* isolates were identified from MacConkey agar plates and biochemically characterized. Four isolates were positive for at least one APEC pentaplex virulence gene (OF8, OF15, OF40, OF43) (Table A2). Strains OF8 (*iut*A) and OF15 (*iro*N) were positive for only one gene each. However, isolates OF40 (*iut*A, *hly*F, *iro*N, *iss* and *omp*T) and OF43 (*iut*A, *hly*F, *iss* and *omp*T) were positive for 5 and 4 genes of APEC pentaplex, respectively.

The *fim*H and *ecp*A genes were found in higher proportions of 89.0% and 81.2%, respectively. The genes *tra*T (32.8%) and *sit*A (6.2%) were also identified. All isolates were negative for the other 8 genes investigated. Description and distribution of genes investigated are shown in Table A2.

All strains were assigned into 4 main phylogenetic groups. Most strains belonged to phylogenetic groups A, with 38 isolates (59.3%), and B1, with 22 isolates (34.3%). The groups B2 and D presented 3 (4.6%) and one (1.5%) isolate, respectively.

Pathogenicity Island Assay

Two multiplex PCRs were performed for each strain. Five PAIs (III536, IV536, IICFT073, ICFT073 and II536) were observed among the *E. coli* strains. PAI I 536 and PAI I J96 were not detected using this assay.

The assay showed that the more prevalent PAI sequence between the positive samples was IICFT073, which was present in 33 strains (51.5%), followed by 13 strains for PAI II536 (20.3%), and 11 strains for PAI III536 (17.1%) ($P < 0.01$). Forty *E. coli* strains were positive for pathogenicity islands (62.5%); among these, 24 strains were positive for only one PAI (60%), 8 strains for 2 PAIs (20%), and 5 strains for 3 PAIs (12.5%). Three strains were positive for 4 PAI sequences (7.5%): OF35 (III536, IICFT073, ICFT073, II536), OF51 and OF54 (III536, IV536, IICFT073, II536) (Table A2).

Adherence to HEp-2 cells and Biofilm Formation

The majority of strains adhered to HEp-2 cells, and 3 adherence patterns were observed: aggregative, aggregative-diffuse and diffuse. Fifty-eight of the 64 strains were adherents (90.6%), 43 strains were adherence aggregative (74.1%), 10 were adherence aggregative-diffuse (17.2%), one was adherence diffuse (1.7%), 4 (6.9%) presented adherence that was not characterized ($P < 0.05$) (Table A2), and 6 were non-adherent.

Of the 64 isolates, 48 (75%) did not produce biofilm. Among strains that produced biofilm, 12 (18.75%) were classified as moderate biofilm producers and only 4 (6.25%) as strong biofilm producers ($P > 0.05$) (Table A2).

Antimicrobial Susceptibility

Resistance to 17 antimicrobials was examined for all 64 *E. coli* isolates, and we observed that 32 (50.0%) isolates from organic fertilizer were resistant to at least one antimicrobial. The majority of isolates were resistant to tetracycline (35.9%), amoxicillin (20.3%), ampicillin (18.7%), streptomycin (17.1%) and trimethoprim-sulfamethoxazole (12.5%). Two isolates were resistant to 5 antimicrobials (OF33 and OF43) and one isolate (OF61) was resistant to 6 antimicrobials. All isolates were susceptible to aztreonam, chloramphenicol, cefotaxime, ciprofloxacin, imipenem, norfloxacin, enrofloxacin, colistin, and polymyxin B.

DISCUSSION

Composting is a process used for the biological stabilization of organic and chemical wastes by the transformation of matter (Bernal et al., 2009). It reduces the number of pathogenic microorganisms by the exposure time, high temperature, and production of antibiotics by microorganisms due to microbial competition (Hahn, 2004).

Commonly, poultry farmers used a simple composting method by stacking the poultry litter near or around the plantation, either uncovered or covered by a polystyrene canvas. The exposure of solid waste compost to temperatures above 60°C decreases the diversity of species (Strom, 1985). Wilkinson and collaborators (2011) showed that the score of *E. coli* in poultry litter was reduced by >99% in 1 h at 55 or 65°C in laboratory conditions. However, Miller and collaborators (2013) showed that 30% of the analyzed samples of final organic fertilizers were positive for *E. coli*. In our study, we isolated *E. coli* from 16 of 40 samples (40%), which corroborates with data obtained by Miller and collaborators (2013).

The relationship between human and avian ExPEC strains has been investigated. Human and avian ExPEC strains harbored similar virulence genes representing a zoonotic risk (Rodriguez-Siek et al., 2005; Ewers et al., 2007; Moulin-Schouleur et al., 2007). In our study, 2 strains (OF 40 and OF43) harbored genes (*iut*A, *hly*F, *iro*N, *iss* and *omp*T) proposed as predictors of APEC virulence by Johnson and collaborators (2008). Four other genes investigated were found among organic fertilizer *E. coli* isolates (*fim*H, *ecp*A, *tra*T, and *sit*A). Type 1 pili (encoded by the *fim*H gene) and *E. coli* common pilus (encoded by *ecp*A gene) can be found in high frequency in *E. coli* strains; furthermore, these virulence factors have an important role in host-pathogen interaction (Blackburn et al., 2009; Schwartz et al., 2013). The expression of *fim*H gene increases UPEC virulence because this adhesin mediates not only bacterial adherence but also the invasion of human bladder epithelial cells (Martinez et al., 2000). *E. coli* common pilus is strongly associated with neonatal meningitis and sepsis samples (Pouttu et al., 2001). The *tra*T gene, inserted into plasmids F or F-like resistance to antibiotics, encodes an outer membrane protein called TraT (Nemeth et al., 1991). It is suggested that this action interferes in the opsonization of the alternative pathway of the complement system, inhibiting phagocytosis (Aguero et al., 1984). Sabri and collaborators (2006) showed that the Sit system of APEC strain χ7122, responsible for iron transport, contributed to the protection against hydrogen peroxide.

In our isolates, we found a high frequency of phylogenetic groups A and B1 and low number of virulence genes. Some studies suggest that ExPEC strains belonging to groups A and B1 generally have few virulence factors, while strains belonging to groups B2 and D harbor a larger number of virulence genes (Ewers et al., 2007; Kobayashi et al., 2011).

Sabaté and collaborators (2006) observed that 20 (40%) of 50 commensal isolates and 93 (93%) of 100 UPEC isolates were positive for PAI markers. In our study, we observed that 60% of the isolates showed at least one PAI sequence, being the most frequent PAI IICFT073 and PAI II536. These PAIs were found on the same frequency in UPEC strains studied by Sabaté and collaborators (2006).

Bacterial colonization is one of the most important stages of the infection processes. In our study, 43 (74.1%) of 64 *E. coli* strains showed aggregative adherence pattern in human epithelial cells; however, only 8 (18.6%) of these strains produced biofilm. These data

did not corroborate with Wakimoto and collaborators (2004), who found 77.4% of strains with aggregative adherence pattern and biofilm production. Furthermore, among the strains with this adherence pattern, 69.7% presented some PAI and 55.8% of the strains were resistant to at least one antimicrobial.

Our isolates showed low resistance rates compared to other countries. Jianguo and collaborators (2012) isolated *E. coli* from chicken feces in eastern farms in China with high resistance rates to sulfamethoxazole, nalidixic acid, tetracycline, streptomycin, ampicillin, trimethoprim-sulfamethoxazole, enrofloxacin, ciprofloxacin, norfloxacin, and chloramphenicol. In Canada, Merchant and collaborators (2012) studied isolates from poultry litter with high resistance rates to tetracycline, ampicillin, and amoxicillin-clavulanic acid. Despite these low resistance rates, the presence of strains resistant to antimicrobials in our study should be an alert regarding the use of these antimicrobials, given the use of tetracycline, beta-lactams, quinolones, and sulfonamides have been prohibited as growth promoters since 2009 by the Ministry of Agriculture, Livestock and Food Supply (Normative Instruction N° 26, July 9, 2009). Antimicrobials used in food-producing animals have caused the emergence, dissemination, and persistence of antimicrobial resistance for both animals and humans, which is a great public health concern (Mellata, 2013).

In summary, we found a low frequency of virulence genes as well as low antimicrobial resistance among avian organic fertilizer isolates. Therefore, these isolates harbor important ExPEC virulence factors and antimicrobial resistance being potential sources of contamination to humans, especially when they are used in organic agriculture for vegetable cultivation, which may lead to a food safety risk.

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REFERENCES

Aguero, M. E., L. Aron, A. G. Deluca, K. N. Timmis, and F. C. Cabello. 1984. A plasmid-encoded outer membrane protein, TraT, enhances resistance of *Escherichia coli* to phagocytosis. Infect. Immun. 46:740–746.

Bernal, M. P., J. A. Alburquerque, and R. Moral. 2009. Composting of animal manures and chemical criteria for compost maturity assessment. A review. Bioresour. Technol. 100:5444–5453.

Blackburn, D., A. Husband, Z. Saldaña, R. A. Nada, J. Klena, F. Qadri, and J. A. Girón. 2009. Distribution of the *Escherichia coli* common pilus among diverse strains of human enterotoxigenic *E. coli*. J. Clin. Microbiol. 47:1781–1784.

Blanco, M., J. E. Blanco, J. Blanco, M. P. Alonso, C. Balsalobre, M. Mourino, C. Madrid, and A. Juárez. 1996. Polymerase chain reaction for detection of *Escherichia coli* strains producing cytotoxic necrotizing factor type 1 and type 2 (CNF1 and CNF2). J. Microbiol. Methods 26:95–101.

Brasil. 1999. Normas para a produção, tipificação, processamento, envase, distribuição, identificação e certificação da qualidade de produtos orgânicos, sejam de origem animal ou vegetal in Instrução Normativa n° 007 de 17 de maio de 1999. Ministério da Agricultura, Pecuária e Abastecimento. Brasil 1999.

Clermont, O., S. Bonacorsi, and E. Bingen. 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl. Environ. Microbiol. 66:4555–4558.

CLSI. 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals Approved Standard. Approved Standard, M31-A3, third ed. vol. third ed. CLSI. M31-A3, CLSI, Wayne, PA, USA.

CLSI. 2012. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, Approved Standard, M07-A9, ninth ed., CLSI, Wayne, PA, USA.

Cravioto, A., R. J. Gross, S. M. Scotland, and B. Rowe. 1979. An adhesive factor found in strains of *Escherichia coli* belonging to the traditional infantile enteropathogenic serotypes. Curr. Microbiol. 3:95–99.

Dozois, C. M., M. Dho-Moulin, A. Brée, J. M. Fairbrother, C. Desautels, and R. Curtiss. 2000. Relationship between the Tsh autotransporter and pathogenicity of avian *Escherichia coli* and localization and analysis of the Tsh genetic region. Infect. Immun. 68:4145–4154.

Ewers, C., G. Li, H. Wilking, S. Kießling, K. Alt, E. M. Antáo, C. Laturnus, I. Diehl, S. Glodde, T. Homeier, U. Böhnke, H. Steinruck, H. C. Phillip, and L. H. Wieler. 2007. Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: How closely related are they? Inter. J. Med. Microbiol. 297:163–176.

Ewing, W. H. 1986. Edwards and Ewing's Identification of Enterobacteriaceae. Elsevier Science Publishing Company, New York.

Furtula, V., E. G. Farrell, F. Diarrassouba, H. Rempel, J. Pritchard, and M. S. Diarra. 2010. Veterinary pharmaceuticals and antibiotic resistance of *Escherichia coli* isolates in poultry litter from commercial farms and controlled feeding trials. Poult. Sci. 89:180–188.

Guan, J., A. Wasty, C. Grenier, and M. Chan. 2007. Influence of temperature on survival and conjugative transfer of multiple antibiotic-resistant plasmids in chicken manure and compost microcosms. Poult. Sci. 86:610–613.

Hahn, L. 2004. Processamento da cama de aviário e suas implicações nos agroecosistemas. Magister. Universidade Federal de Santa Catarina, Florianópolis, Brazil.

Jianguo, Y., C. Weihua, W. Huafu, and Z. Wei. 2012. Antimicrobial susceptibility and virulence factors of *Escherichia coli* isolates obtained from faeces samples of chickens in east China. Afric. J. Microbiol. Res. 6:1591–1596.

Johannessen, G. S., R. B. Frøseth, L. Solemdal, J. Jarp, Y. Wasteson, and L. M. Rørvik. 2004. Influence of bovine manure as fertilizer on the bacteriological quality of organic iceberg lettuce. J. Appl. Microbiol. 96:787–794.

Johnson, J. R., and A. L. Stell. 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J. Infect. Dis. 181: 261–272.

Johnson, T. J., Y. Wannemuehler, C. Doetkott, S. J. Johnson, S. C. Rosenberger, and L. K. Nolan. 2008. Identification of minimal predictors of avian pathogenic *Escherichia coli* virulence for use as a rapid diagnostic tool. J. Clin. Microbiol. 46:3987–3996.

Kaper, J. B., J. P. Nataro, and H. L. Mobley. 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. 2:123–140.

Kobayashi, R. K., I. Aquino, A. L. Ferreira, and M. C. Vidotto. 2011. EcoR phylogenetic analysis and virulence genotyping of avian pathogenic *Escherichia coli* strains and *Escherichia coli* isolates from commercial chicken carcasses in southern Brazil. Food. Pathog. Dis. 8:631–634.

Le Bouguenec, C., M. Archambaud, and A. Labigne. 1992. Rapid and specific detection of the *pap*, *afa*, and *sfa* adhesin-encoding

operons in uropathogenic *Escherichia coli* strains by polymerase chain reaction. J. Clin. Microbiol. 30:1189–1193.

Martinez, J. J., M. A. Mulvey, J. D. Schilling, J. S. Pinkner, and S. J. Hultgren. 2000. Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. EMBO J. 19:2803–2812.

Mellata, M., M. Dho-Moulin, C. M. Dozois, R. Curtiss, P. K. Brown, P. Arne, A. Brée, C. Desautels, and J. M. Fairbrother. 2003. Role of virulence factors in resistance of avian pathogenic *Escherichia coli* to serum and in pathogenicity. Infect. Immun. 71: 536–540.

Mellata, M. 2013. Human and avian extraintestinal pathogenic *Escherichia coli*: infections, zoonotic risks, and antibiotic resistance trends. Food. Pathog. Dis. 10:916–932.

Merchant, L. E., H. Rempel, T. Forge, T. Kannangara, S. Bittman, P. Delaquis, E. Topp, K. A. Ziebell, and M. S. Diarra. 2012. Characterization of antibiotic-resistant and potentially pathogenic *Escherichia coli* from soil fertilized with litter of broiler chickens fed antimicrobial-supplemented diets. Can. J. Microbiol. 58:1084–1098.

Miller, C., S. Heringa, J. Kim, and X. Jiang. 2013. Analyzing indicator microorganisms, antibiotic resistant *Escherichia coli*, and regrowth potential of foodborne pathogens in various organic fertilizers. Food. Pathog. Dis. 10:520–527.

Moulin-Schouleur, M., M. Reperant, S. Laurent, A. Bree, S. Mignon-Grasteau, P. Germon, D. Rasschaert, and C. Schouler. 2007. Extraintestinal pathogenic *Escherichia coli* strains of avian and human origin: link between phylogenetic relationships and common virulence patterns. J. Clin. Microbiol. 45: 3366–3376.

Nataro, J. P, J. B. Kaper, R. Robins-Browne, V. Prado, P. Vial, and M. M. Levine. 1987. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. Pediat. Infect. Dis. J. 6: 829–831.

Nemeth, J., C. A. Muckle, and R. Y. C. Lo. 1991. Serum resitance and the *tra*T gene in bovine mastitis-causing *Escherichia coli*. Vet. Microbiol. 28:343–351.

Oliveira, M., J. Usall, I. Vinas, M. Anguera, F. Gatius, and M. Abadias. 2010. Microbiological quality of fresh lettuce from organic and conventional production. Food. Microbiol. 27: 679–684.

Poole, T. L., J. L. McReynolds, T. S. Edrington, J. A. Byrd, T. R. Callaway, and D. J. Nisbet. 2006. Effect of flavophospholipol on conjugation frequency between *Escherichia coli* donor and recipient pairs in vitro and in the chicken gastrointestinal tract. J. Antimicrob. Chemother. 58:359–366.

Poppe, C., L. C. Martin, C. L. Gyles, R. Reid-Smith, P. Boerlin, S. A. McEwen, J. F. Prescott, and K. R. Forward. 2005. Acquisition of resistance to extended-spectrum cephalosporins by *Salmonella enterica* subsp. enterica serovar Newport and *Escherichia coli* in the turkey poult intestinal tract. Appl. Environ. Microbiol. 71:1184–1192.

Pouttu, R., B. Westerlund-Wikström, H. Lång, K. Alsti, R. Virkola, U. Saarela, A. Siitonen, N. Kalkkinen, and T. K. Korhonen. 2001. *matB*, a common fimbrillin gene of *Escherichia coli*, expressed in a genetically conserved, virulent clonal group. J. Bacteriol. 183:4727–4736.

Puño-Sarmiento, J., L. E. Gazal, L. P. Medeiros, E. K. Nishio, R. K. T. Kobayashi, and G. Nakazato. 2014. Identification of diarrheagenic *Escherichia coli* strains from avian organic fertilizers. Int. J. Environ. Res. Public Health 11:8924–8939.

Rodriguez-Siek, K. E., C. W. Giddings, C. Doetkott, T. J. Johnson, M. K. Fakhr, and L. K. Nolan. 2005. Comparison of *Escherichia coli* isolates implicated in human urinary tract infection and avian colibacillosis. Microbiol. 151:2097–2110.

Russo, T. A., C. D. McFadden, U. B. Carlino-MacDonald, J. M. Beanan, T. J. Barnard, and J. R. Johnson. 2002. IroN functions as a siderophore receptor and is a urovirulence factor in an extraintestinal pathogenic isolate of *Escherichia coli*. Infect. Immun. 70:7156–7160.

Sabaté, M., E. Moreno, T. Perez, A. Andreu, and G. Prats. 2006. Pathogenicity island markers in commensal and uropathogenic *Escherichia coli* isolates. Clin. Microbiol. Infect. 12: 880–886.

Sabri, M., S. Léveillé, and C. M. Dozois. 2006. A SitABCD homologue from an avian pathogenic *Escherichia coli* strain mediates transport of iron and manganese and resistance to hydrogen peroxide. Microbiol. 152:745–758.

Santos, T. M. B., J. J. Lucas, and N. K. Sakomura. 2005. Effects of broiler stocking density and poultry litter reuse in broiler performance and poultry litter production. Rev. Port. Ciên. Vet. 553–554:45–52.

Scaletsky, I. C., J. Michalski, A. G. Torres, M. V. Dulguer, and J. B. Kaper. 2005. Identification and characterization of the locus for diffuse adherence, which encodes a novel afimbrial adhesin found in atypical enteropathogenic *Escherichia coli*. Infect. Immun. 73:4753–4765.

Schwartz, D. J., V. Kalas, J. S. Pinkner, S. L. Chen, C. N. Spaulding, K. W. Dodson, and S. J. Hultgren. 2013. Positively selected FimH residues enhance virulence during urinary tract infection by altering FimH conformation. Proc. Natl. Acad. Sci. USA. 110:15530–15537.

Strom, P. F. 1985. Effect of temperature on bacterial species diversity in thermophilic solid-waste composting. Appl. Environ. Microbiol. 50:899–905.

Sweeten, J. M.,, and B. W. Auvermann. 2008. Composting Manure and Sludge. Agrilife Extension. 479 ed. The Texas A&M University System, Colorado, TX, pp. 06–08.

Toledo, M. R. F., C. F. Fontes, and L. R. Trabulsi. 1982a. EPM – a modification of Rugai and Araujo medium for simultaneous test of gas production from glucose, H_2S, urease and tryptophan deaminase. Rev. Microbiol. 13:309–315.

Toledo, M. R. F., C. F. Fontes, and L. R. Trabulsi. 1982b. MILi – a medium for detection of motilily, indole, and lysine decarboxylase. Rev. Microbiol. 13:230–235.

UBABEF. 2014. Annual Report of Brazilian Poultry Association. Brazilian Poultry Union, São Paulo, Brazil.

Wakimoto, N., J. Nishi, J. Sheikh, J. P. Nataro, J. Sarantuya, M. Iwashita, K. Manago, K. Tokuda, M. Yoshinaga, and Y. Kawano. 2004. Quantitative biofilm assay using a microtiter plate to screen for enteroaggregative *Escherichia coli*. Am. J. Trop. Med. Hyg. 71:687–690.

Wilkinson, K. G., E. Tee, R. B. Tomkins, G. Hepworth, and R. Premier. 2011. Effect of heating and aging of poultry litter on the persistence of enteric bacteria. Poult. Sci. 90: 10–18.

Yamamoto, S., A. Terai, K. Yuri, H. Kurazono, Y. Takeda, and O. Yoshida. 1995. Detection of urovirulence factors in *Escherichia coli* by multiplex polymerase chain reaction. FEMS Immun. Med. Microbiol. 12:85–90.

Zhong, W., T. Gu, W. Wang, B. Zhang, X. Lin, Q. Huang, and W. Shen. 2010. The effects of mineral fertilizer and organic manure on soil microbial community and diversity. Plant. Soil 326:523–523.

APPENDIX

Table A1. Primer sequence, size of gene and reference for virulence factors, phylogenetic groups and pathogenicity island of *E. coli* from organic fertilizer.

Primers	Primer sequence (5′- 3′)	Size of gene	Reference
Pathogenicity islands			
PAI I536	TAATGCCGGAGATTCATTGTC AGGATTTGTCTCAGGGCTTT	1.800	Sabaté et al., 2006
PAI II536	CTACGTCAGGCTGGCTTTG TCGTGCTCAGGTCCGGAATTT	1.000	Sabaté et al., 2006
PAI III 536	CGGGCATGCATC AATTATCTTTG TGTGTAGATGCAGTCACTCCG	200	Sabaté et al., 2006
PAI IV 536	AAGGATTCGCTGTTACCGGAC TCGTCGGGCAGCGTTTCTTCT	300	Sabaté et al., 2006
PAI ICFT073	GGACATCCTGTTACAGCGCGCA TCGCCACCAATCACAGCGAAC	930	Sabaté et al., 2006
PAI IICFT073	ATGGATGTTGTATCGCGC ACGAGCATGTGGATCTGC	400	Sabaté et al., 2006
PAI IJ96	CATGTCCAAAGCTCGAGCC TGGCATCCCACATTATCG	400	Sabaté et al., 2006
Phylogenetic groups			
*chu*A	GACGAACCAACGGTCAGGAT TGCCGCCAGTACAAAGACA	279	Clermont et al., 2000
*yja*A	TGAAGTGTCAGAGACGCTG ATGGAGAATGCGTTCCTCAAC	211	Clermont et al., 2000
TspE4C2	GAGTAATGTCGGGGCATTCA CGCGCCAACAAAGTATTACG	152	Clermont et al., 2000
Virulence factors			
*iro*N	AATCCGGCAAAGAGACGAACCGCCT GTTCGGGCAACCCCTGCTTTGACTTT	553	Johnson et al., 2008
*omp*T	TCATCCCGGAAGCCTCCCTCACTACTAT TAGCGTTTGCTGCACTGGCTTCTGATAC	496	Johnson et al., 2008
*hly*F	GGCCACAGTCGTTTAGGGTGCTTACC GGCGGTTTAGGCATTCCGATACTCAG	450	Johnson et al., 2008
iss	CAGCAACCCGAACCACTTGATG AGCATTGCCAGAGCGGCAGAA	323	Johnson et al., 2008
*iut*A	GGCTGGACATCATGGGAACTGG CGTCGGGAACGGGTAGAATCG	302	Johnson et al., 2008
*ecp*A	TGAAAAAAAAAGGTTCTGGCAATAGC CGCTGATGAGGAGAAAGTGAA	483	Blackburn et al., 2009
tsh	GGTGGTGCACTGGAGTGG AGTCCAGCGTGATAGTGG	640	Dozois et al., 2000
*hly*A	AAC AAG GAT AAG CAC TGT TCT GGC ACC ATA TAA GCG GTC ATT CCC GTC	1177	Yamamoto et al., 1995
*cnf*1	AGGATGGAG TTT CCT ATGCAGGAG CAT TCA GAG TCC TGC CCT CAT TAT T	498	Yamamoto et al., 1995
*cnf*2	AAT CTA ATT AAA GAG AAC CAT GCT TTG TAT ATC TA	543	Blanco et al., 1996
*ibe*A	AGG CAG GTG TGC GCC GCG TAC TGG TGC TCC GGC AAA CCA TGC	170	Johnson and Stell, 2000
*fyu*A	TGA TTA ACC CCG CGA CGG AA CGC AGT AGG CAC GAT CTT GTA	880	Johnson and Stell, 2000
*sit*A	AGGGGGCACAACTGATTCTCG TACCGGGCCGTTTTCTGTGC	608	Johnson and Stell, 2000
*tra*T	GGT GTG GTG CGA TGA GCA CAG CAC GGT TCA GCC ATC CCT GAG	290	Johnson and Stell, 2000
*pap*C	GAC GGC TGT ACT GCA GGG TGT GGC G ATA TCC TTT CTG CAG GCA GGG TGT GGC	328	Le Bouguenec et al., 1992
*pap*G	CTG TAA TTA CGG AAG TGA TTT CTG ACT ATC CGG CTC CGG ATA AAC CAT	1070	Johnson and Stell, 2000
fimH	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA	508	Johnson and Stell, 2000

Table A2. Genotypic and phenotypic characteristics of *E. coli* isolates from organic fertilizer.

Isolate	Virulence genes of ExPEC	Phylogenetic group	Pattern of adherence HEp-2	Phenotype of resistance	Biofilm	Island of pathogenicity
OF 3	*fimH*/*ecp*A	A	AA	–	STRONG	–
OF 4	*fimH*	A	AA	NAL/AMO/STR	WEAK	–
OF 5	*tra*T/*fimH*/*ecp*A	A	AA/DA	–	NON-BP	IICFT073
OF 6	*tra*T/*fimH*/*ecp*A	A	AA	–	WEAK	IICFT073
OF 11	*ecp*A	A	AA	AMO/AMP/TET	NON-BP	IV536/ICFT073
OF 12	*tra*T/*fimH*/*ecp*A	A	NA	AMO	NON-BP	IICFT073/II536
OF 13	*tra*T/*fimH*/*ecp*A	A	NA	–	NON-BP	IICFT073
OF 14	*tra*T/*fimH*/*ecp*A	A	NA	–	NON-BP	IICFT073
OF 15	*Iro*N	A	AA	AMO/AMC/AMP	WEAK	IICFT073
OF 19	*fimH*	A	AA	NAL/SXT	NON-BP	–
OF 20	*ecp*A	A	NC	–	STRONG	IV536
OF 21	–	A	AA	–	NON-BP	IV536/IICFT073
OF 22	*fimH*/*ecp*A	A	AA	–	STRONG	IICFT073
OF 24	*fimH*/*ecp*A	A	AA	–	NON-BP	IICFT073/II536
OF 29	*fimH*/*ecp*A	A	NC	AMO/AMP/TET	WEAK	II536
OF 30	*fimH*/*ecp*A	A	NC	TET	NON-BP	–
OF 31	*fimH*	A	NA	NAL/SXT/TET	WEAK	III536/IICFT073/II536
OF 32	*fimH*	A	AA	TET	NON-BP	III536/IICFT073/II536
OF 33	*sit*A/*tra*T/*fimH*	A	AA	AMO/AMP/STR/SXT/TET	NON-BP	III536/IICFT073/ICFT073
OF 34	*tra*T/*fimH*	A	AA	–	STRONG	III536/IICFT073/II536
OF 35	*fimH*	A	AA	TET	NON-BP	III536/IICFT073/ICFT073/II536
OF 37	*fimH*/*ecp*A	A	AA/DA	–	WEAK	–
OF 38	*fimH*/*ecp*A	A	NC	–	NON-BP	–
OF 40	*sit*A/*tra*T/*iro*N/*hly*F/*iut*A/*iss*/*omp*T/*fimH*/*ecp*A	A	AA	AMP/STR/SXT/TET	NON-BP	–
OF 41	*fimH*/*ecp*A	A	AA/DA	–	NON-BP	–
OF 42	*fimH*/*ecp*A	A	AA	–	NON-BP	–
OF 44	*fimH*/*ecp*A	A	AA	STR/SXT/TET	NON-BP	–
OF 46	*tra*T/*fimH*/*ecp*A	A	AA	STR	NON-BP	–
OF 49	*fimH*/*ecp*A	A	AA	–	NON-BP	–
OF 53	*fimH*/*ecp*A	A	AA	STR/TET	NON-BP	ICFT073/II536
OF 55	*fimH*/*ecp*A	A	AA	NAL/SXT/TET	NON-BP	III536/IICFT073
OF 56	*tra*T/*fimH*/*ecp*A	A	AA	NAL/SXT/TET	NON-BP	III536/IICFT073/ICFT073
OF 57	*fimH*/*ecp*A	A	AA	–	NON-BP	IICFT073
OF 58	*fimH*	A	AA	TET	NON-BP	III536/IICFT073
OF 59	*ecp*A	A	AA/DA	AMO/AMP/STR/TET	NON-BP	IICFT073
OF 60	*fimH*	A	AA	–	NON-BP	IICFT073
OF 61	*fimH*	A	AA	NAL/AMO/AMC/AMP/STR/TET	NON-BP	IICFT073
OF 63	*ecp*A	A	AA	–	NON-BP	IICFT073
OF 1	*tra*T/*fimH*/*ecp*A	B1	AA	AMO/AMP/TET	NON-BP	–
OF 2	*fimH*/*ecp*A	B1	AA/DA	AMO/AMP	WEAK	–
OF 7	*tra*T/*fimH*/*ecp*A	B1	NA	–	WEAK	IICFT073
OF 8	*sit*A/*tra*T/*iut*A/*fimH*/*ecp*A	B1	AA	AMC	NOM-BP	IICFT073
OF 9	*tra*T/*fimH*/*ecp*A	B1	AA	AMP	NON-BP	IICFT073
OF 10	*tra*T/*fimH*/*ecp*A	B1	AA	AMO	NON-BP	IICFT073
OF 16	*tra*T/*fimH*/*ecp*A	B1	AA	STR/GEN/TET	NON-BP	IICFT073
OF 17	*fimH*/*ecp*A	B1	AA	–	WEAK	–
OF 18	*tra*T/*fimH*/*ecp*A	B1	AA/DA	–	NON-BP	–
OF 23	*fimH*/*ecp*A	B1	AA	–	WEAK	IICFT073
OF 27	*fimH*/*ecp*A	B1	AA	–	NON-BP	II536
OF 28	*fimH*/*ecp*A	B1	AA/DA	–	NON-BP	III536/IICFT073
OF 39	*tra*T/*fimH*/*ecp*A	B1	AA/DA	–	NON-BP	–
OF 45	*fimH*/*ecp*A	B1	NA	AMO/AMP/TET	NON-BP	–
OF 47	*tra*T/*fimH*/*ecp*A	B1	AA	–	NON-BP	–
OF 50	*fimH*/*ecp*A	B1	AA	STR/TET	NON-BP	II536
OF 51	*fimH*/*ecp*A	B1	AA	TET	NON-BP	III536/IV536/IICFT073/II536

Table A2 *Continued.*

Isolate	Virulence genes of ExPEC	Phylogenetic group	Pattern of adherence HEp-2	Phenotype of resistance	Biofilm	Island of pathogenicity
OF 52	*fimH*/*ecp*A	B1	AA	TET	NON-BP	II536
OF 54	*fimH*/*ecp*A	B1	AA	TET	NON-BP	III536/IV536/IICFT073/ II536
OF 62	*fimH*/*ecp*A	B1	AA	–	NON-BP	–
OF 64	*ecp*A	B1	AA	–	NON-BP	IV536
OF 25	*fimH*/*ecp*A	B2	AA	–	NON-BP	IICFT073
OF 26	*tra*T/*fimH*/*ecp*A	B2	DA	–	NON-BP	–
OF 36	*fimH*/*ecp*A	B2	AA	–	NON-BP	–
OF 48	*fimH*/*ecp*A	B2	AA/DA	–	NON-BP	–
OF 43	*sit*A/*tra*T/*hly*F/*iut*A/*iss*/*omp*T/*fimH*/*ecp*A	D	AA/DA	AMO/AMP/STR/SXT/TET	NON-BP	–

Biofilm formation: NON-BF - No biofilm formed; STRONG - strong adherence; WEAK - weak adherence.

Adherence patterns: AA - Aggregative; DA - Diffuse; AA/DA - Aggregative-Diffuse; NC - Non-Characteristic; NA - Non-Adherence.

Antimicrobial: amoxicillin (AMO); amoxicillin-clavulanic acid (AMC); ampicillin (AMP); aztreonam (ATM); cefotaxime (CTX); imipenem (IPM); tetracycline (TET); gentamicin (GEN); chloramphenicol (CHL); trimethoprim-sulfamethoxazole (SXT); nalidixic acid (NAL); ciprofloxacin (CIP); norfloxacin (NOR); streptomycin (STR); enrofloxacin (ENR); colistin (COL); and polymyxin B (POL).