Highly Pathogenic and Multidrug Resistant Avian Pathogenic Escherichia Coli in Free-Range Chickens from Brazil

ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) virulence mechanism has been continuously studied and it is believed to be multifactorial and because of this, this work aimed to characterize potentially APEC strains isolated from free-range hens. Isolates were submitted to PCR for the detection of virulence genes, which were of high prevalence. *In vivo* inoculation of day-old chicks revealed that 49 of these strains were of high and intermediate pathogenicity. In addition, isolates were submitted to antimicrobials susceptibility test with the majority of the strains presenting multiresistance. Phylogenetic analysis showed a greater presence of potentially APEC isolates in-group B2. In addition, high heterogeneity was detected among the isolates by XbaI enzyme. Fifteen serogroups were identified, being the O8 the most frequent. These results strengthen the fact that a combination of diverse factors are associated with the pathogenicity APEC strains, as well as to highlight its importance to public health and that free-range hens can act as a reservoir of potentially zoonotic bacteria.

INTRODUCTION

Small producers in the word perform mainly free-range hens raising, with precarious installations and minimal management often being its main limiting factors. These practices may contribute to the spread of diseases to both birds and consumers (Thekisoe *et al.*, 2003). In this regard, the avian pathogenic *Escherichia coli* (APEC) is a major agent with increasing interest among avian sanity, being associated with a series of extra-intestinal systemic infections, collectively referred as colibaciloses (Kaper *et al.*, 2004) and it is responsible for economic losses on poultry industry (Dho-moulin & Fairbrother, 1999).

*E. coli* strains pathogenicity are related to virulence factors that are used to differentiate between pathogenic and non-pathogenic strains (Rodriguez-Siek *et al.*, 2005). In this regard, a large number of potential virulence factors have been detected; however, virulence mechanisms have not yet been fully elucidated and, thus, require further studies. There is still no consensus in the literature as to which genes would be the ideal virulence markers. So far, it has been shown that APEC strains present virulence genes, which can be translated into adhesins, toxins, siderophores, colicin, serum resistance and others (Barbieri *et al.*, 2013).

However, it is known that the zoonotic potential of APEC strains is evidenced when common virulence factors of APEC are found in *E. coli* strains, thus resulting in extra intestinal diseases in humans. This characterize a positive relationship between the APEC, UPEC and NMEC (Johnson *et al.*, 2008). Hens and humans often share the same environment; there, these birds may present an important source of human infection, as well as acquire human strains. Glimpsing the difficulty to define APEC pathotype, this work aims to evaluate possible
genes related to APEC virulence and analyze the phenotype of isolates obtained from free-range hens raising in Brazil.

MATERIAL AND METHODS

This experiment was approved by the Committee on Ethics for the Use of Animals of the São Paulo State University (Unesp), School of Agricultural and Veterinary Sciences, São Paulo, Brazil, under protocol number 05749/14.

Population analyzed and Collection of samples

Samples were collected from 250 hens of unknown genetic origin and different age from seven small farms within Ribeirão Preto region, Sao Paulo state, Brazil, from January to April 2014. Five hundred samples were obtained, being 250 from the cloaca and 250 from the oropharynges. After collection, samples were placed in tubes containing 5 mL of BHI broth and kept on ice until arrival at the laboratory.

Detection of pathotypes and virulence genes

In order to detect APEC, a PCR screening was performed for the cvaC, iroN, iss, iutA, ompT and hlyF genes. Samples that were positive for at least five of the genes were used to detect E. coli isolates as recommended by Kemmett et al. (2013). DNA template preparation as well as PCR procedures and primers were used according to the Ecl protocol available at http://www.apzec.ca/en/APZEC/Protocols/APZEC_PCR_en.aspx. In addition, all isolates were evaluated for 11 additional virulence genes as follow: sitA, tsh, traT, vat, astA, iucC, iucD, papC, ipr2, fimH and fyuA; also following the Ecl protocol as cited above.

Serological identification and antimicrobial susceptibility test

Serotyping was performed by plate agglutination procedure according to Orskov et al. (1977) at the “E. coli Reference Center” (ECRC) at the Pennsylvania State University - USA. Serology was carried out using serogroups O1-O181 antisera with the exceptions of O31, O47, O72, O93, O94, and O122. Additionally, isolates were submitted, by the disc diffusion method (CLSI, 2010), to antimicrobial susceptibility testing against the following: ampicillin (10μg), cephalothin (30μg), streptomycin (10μg), gentamicin (10μg), ciprofloxacin (5μg), chloramphenicol (30μg), tetracycline (30 μg), nitrofurantoin (300μg), sulfamethoxazole + trimethoprim (25μg), cefitoxin (30μg), ceftriaxone (30μg), amoxicillin + clavulanic acid (30μg), norfloxacin (10μg) and fosfomycin (50μg).

Phylogenetic Typing and Pulsatile Field Electrophoresis (PFGE)

Identification of chuA and yjaA genes and TspE4. C2 DNA fragment was performed with the primers proposed by Clermont et al. (2000). Genomic DNA digestion with XbaI and plug preparation was done as described by Ribot et al. (2006) with modifications and Salmonella strain Braenderup H9812 was used as a molecular weight reference. Migration was performed on 1% Pulsfield certified agarose gel with an initial time of 2.2 seconds and final time of 54.2 seconds on a 6 V cm-1 gradient and 120° angle for 23h at a temperature of 14°C. Similarity analysis was performed using Dice coefficients with 1% band position tolerance and 0.5% optimization. In addition, a dendrogram was obtained by UPGMA. These analysis were performed with the BioNumerics software version 7.1 (Applied Maths, Sint-Martens-Latem, Belgium).

Pathogenicity test

A 0.1 ml of bacterial culture were inoculated into 10 ml of BHI broth, incubated for 18 hours at 37 °C and subsequently diluted to a 1:10 ratio. Inoculum concentration was standardized to 10⁵ CFU/mL. The E. coli (serogroup O1) belonging to the Laboratory of Ornithopathology of USP, was used as a positive control. Negative control birds were inoculated with BHI broth only. For each strain, as well as for the negative and positive control groups, ten male chicks from a commercial lineage were used. The strains were classified due to its mortality as follow: high (≥ 80%), intermediate (> 50% and <80%), low pathogenicity (≤ 50%) and non-pathogenic (zero mortality).

RESULTS AND DISCUSSION

Detection of additional pathotypes and virulence genes

From the 500 samples screened by PCR, 139 (27.8%) samples were positive for at least five of the APEC related genes (cvaC, iroN, iss, iutA, ompT and hlyF), of these, 75 were from the cloaca and 64 from the oropharynx. Of these positive samples, 69 (49.6%) strains (36 cloaca and 33 oropharynx) were isolated. The frequency of the referred genes observed in E. coli isolates and other virulence genes is shown in Table 1.
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Table 1 – Frequency of each gene associated with virulence in the potentially 69 isolates of APEC from free-range hens.

| Function       | Genes   | Frequency (%) |
|----------------|---------|---------------|
| Adhesion       | fimH    | 82.6          |
|                | papC    | 1.5           |
|                | tsh     | 30.4          |
| Iron acquisition| fyuA   | 39.1          |
|                | iroN    | 87.0          |
|                | iucC    | 58.0          |
|                | iucD    | 74.0          |
|                | irp2    | 76.8          |
|                | iutA    | 88.4          |
|                | sitA    | 89.8          |
| Haemolysin     | hlyF    | 100           |
| Serum resistance| iss    | 100           |
|                | traT    | 82.6          |
| Toxins         | astA    | 15.9          |
|                | vat     | 17.4          |
| Multiple functions| cvaC  | 66.7          |
|                | ompT    | 100           |

High percentages (75.4%) of all six genes were observed, and according to Rodriguez-siek et al. (2005), traT gene associated with the cvaC gene in the APEC strains make part of the serum effects resistance mechanism and are usually associated with septicemia. In accordance with the definition of Ewers et al. (2007) for an isolate to be considered pathogenic the presence of at least one adhesion factor is necessary, one of iron acquisition and one of serum resistance. The present study observed that 64 (92.7%) isolates from free-range chicken harbored at least one adhesion-related virulence factor, one iron acquisition factor and a serum resistance factor. Serum resistance, is mainly encoded by the iss gene (Monroy et al., 2005), and was found in 100.0% of the isolates and according to Tivendale et al. (2004) the iss gene was related to high levels of virulence and some authors report it is of high prevalence. In an interesting manner, the present study detected a frequency of 15.9% of the astA gene, with a similar frequency of the study of Won et al. (2009) of 17.8%. However, it is lower than the 20.0% frequency observed by Ewers et al. (2004). These differences may be, according to this author, due to this gene being present in a pathogenicity island.

Antimicrobial susceptibility test

All 69 tested isolates demonstrated resistance to at least one antimicrobial agent and most of them showed a multi-drug resistance profile with 59 (85.5%) isolates simultaneous resistant to three or more antimicrobials (Figure 1). It is often common that birds E. coli isolates present resistance to more than one antimicrobial, being the main reasons the indiscriminate and prolonged use of sub-therapeutic concentrations and inadequate use antimicrobial therapies (Mellata et al., 2013). Some studies carried out in Brazil report that APEC strains resist to all classes of drugs, with sulfonamides and tetracyclines having the highest indexes, ranging from 50.0% to 90.0% (Zanatta et al., 2004). Accordingly, the present study verified isolates with high resistance for tetracycline (69.5%) and sulfonamides (58.3%). In addition, among the aminoglycosides tested, streptomycin presented a higher index (63.8%). An explanation for these high levels of resistance resides in the fact that 100% of the strains were iss positive. This gene, in addition to the increased serum resistance, may lead to resistance to various antimicrobials (Abreu et al., 2010) and can be transferred, by conjugation, to other nonvirulent bacteria, including ones of different species thus they become more pathogenic and resistant (Johnson et al., 2006).
Phylogenetic Typing

The analysis revealed that most of the isolates in this study belong to phylogenetic group B2 (37/69), followed by group A (17/69), group B1 (13/69) and group D (2/69), as shown in Table 2. Studies of phylogenetic analyzes have shown that *E. coli* isolates can be grouped into four main phylogenetic groups: A, B1, B2 and D. Extra-intestinal pathogenic samples with a large variety of virulence factors are concentrated in groups B2 and D. Meanwhile, commensal samples are concentrated in groups A and B1 (Le Gall et al., 2007). Isolates of phylogenetic groups B2 and D were associated with a greater number of virulence factors, presenting a mean of 11.5 and 13.5 virulence factors per isolate, respectively. Phylogenetic groups A and B1 presented 9.9 and 10.8 virulence factors per isolate, respectively. Studies suggest that virulent clonal groups are mainly derived from phylogenetic group B2 and, to a lesser extent, from group D, explaining the presence and absence of virulence genes between these pulses could be explained by the presence of a capsule that are found in virulent APEC strains (Moulin-schouleur et al., 2006) and to genetic changes either caused by mutation (chromosome alteration) or by genetic transfer (Skyberg et al., 2003).

Table 2 – Distribution of the 69 potentially APEC isolate in correlation with the phylogenetic group.

| Phylogenetic group (nº - %) | VF mean/isolate* |
|-----------------------------|------------------|
| A (17 – 24.6%)              | 9.9              |
| B1 (13 – 18.8%)             | 10.8             |
| B2 (37 – 53.6%)             | 11.5             |
| D (2 – 2.9%)                | 13.5             |

*Represents a sum total of the virulence factors of the group and divided by the number of isolates.

Pathogenicity

Forty-three (62.3%) strains were highly pathogenic, six (8.7%) strains were intermediate pathogenic, 16 (23.2%) strains were low pathogenic, and four (5.8%) were non-pathogenic. All positive control birds deceased, while negative control birds remained alive. Clinical signs and macroscopic lesions were observed in higher frequency among birds inoculated with the high and intermediate pathogenic strains. Guastalli et al. (2013) in a study with commercial laying hens that showed signs of colibacillosis obtained approximately 50.0% of their isolates with high or intermediate pathogenicity, frequency below our result. This can be explained by the fact that free-range chickens have a higher genetic variability and greater rusticity, which

Pulsatile Field Electrophoresis (PFGE)

Of the 69 potentially APEC isolates, only one was not typable by the XbaI enzyme with the remaining 68 isolates generating 59 pulse types. All other isolates were grouped into single pulse types, demonstrating a high degree of heterogeneity is present among the APECs examined with the generated dendrogram by PFGE presenting three large clusters (Figure 2). Six pulse types were shared by more than one isolate, with 100% similarity. The pulse types 1Ca3 and 5Ca1, 21Fa4 and 71Fa1, 37Ca1 and 71Fa5 belonged to the same property. This could be explained by the fact that the birds were in constant contact in almost all the properties visited facilitating the transmission of clones. The pulse types 52Ca1 and 81Ca3, 52Ca5 and 82Fa1, 56Ca4 and 88Ca4 from free-range hens from different locations present different genotypic profiles, pathogenicity and phylogenetic typing with only the last pulsetype presenting different serogroups, and the first two being non-typable for the O antigen. Pulsetypes with similarity greater than 95.0% were also found within the same bird. The isolates were from samples collected from the oropharynx, being:

149Fa3 and 149Fb2, 175Fa1 and 175Fb1, and 236Fa1 presenting genotype and phenotype similarities, except for pulse 149Fa3 and 149Fb2, which although were from the same bird, presented in the in vivo test, quite distinct results, being of low and high pathogenicity. Differences between the presence and absence of virulence genes between these pulses could be explained by the presence of a capsule that are found in virulent APEC strains (Moulin-schouleur et al., 2006) and to genetic changes either caused by mutation (chromosome alteration) or by genetic transfer (Skyberg et al., 2003).
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gives them resistance to diseases, adverse climate and food conditions (Albino et al., 2001). Interestingly, in this study, 29.0% of the low or non-pathogenic isolates presented a high number of genes related to APEC virulence. According to Ikuno et al. (2006), the presence of a virulence gene in commensal E. coli strains can be used as an indicator of potential risks, but it is necessary to investigate beyond the presence of the genes and look for their expression. Thus, it can be concluded that, even if healthy, the “backyard chickens” are carriers and can disseminate pathogenic E. coli strains, of which can be transferred to other birds and/or animals, thus representing an important source of infection or reservoir.

Although APEC is not pathogenic to humans, it is of concern that poultry samples presents similarities to those of humans and that most virulence genes are similar to the ones identified in extra-intestinal strains causing diseases in humans, thus representing zoonotic risk (Johnson & Russo, 2002). The results found in the present study showed that hens act as reservoirs of multi-drug resistant and highly pathogenic ExPEC, representing a risk to the consumer, mainly because these birds live in close proximity to humans and other animals. It is important to emphasize that animals with virulence factors are an important source of infection, since the bacteria can be excreted along with feces or expelled by the respiratory tract. This is reinforced by the fact that although the isolates were obtained from samples of apparently healthy hens, and that, in principle, should be non-pathogenic; more than 70.0% were of high or intermediate pathogenicity by the test performed in 1 day-old chicks. Therefore, APEC studies in free-range hens, that can act as reservoirs and disseminators of this pathogen, contribute to improve methods for diagnosis, control, prevention and treatment of the diseases caused by this bacterium.
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