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Classification of Avian Pathogenic *Escherichia coli* by a Novel Pathogenicity Index Based on an Animal Model

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ABSTRACT

Background: Avian Pathogenic *Escherichia coli* is the main agent of colibacillosis, a systemic disease that causes considerable economic losses to the poultry industry. *In vivo* experiments are used to measure the ability of *E. coli* to be pathogenic. Generally, these experiments have proposed different criteria for results interpretation and did not take into account the death time. The aim of this study was to propose a new methodology for the classification of *E. coli* pathogenicity by the establishment of a pathogenicity index based in the lethality, death time and the ability of the strain to cause colibacillosis lesions in challenged animals.

Materials, Methods & Results: A total of 293 isolates of *E. coli* were randomly selected to this study. The strains were isolated from cellulitis lesions, broiler bedding material or respiratory diseases and were previously confirmed through biochemical profile. The bacterial isolates were kept frozen at -20°C. The strains were retrieved from stocks and cultured in brain-heart infusion broth overnight at 37°C to obtain a final concentration of 10^9 UFC/mL. A total of 2940 one-day-old chicks from commercial breeding hens were randomly assigned to groups containing 10 animals and each group was subcutaneously inoculated in the abdominal region with 0.1 mL of the standard inoculum solution containing each of the strains. A control group of 10 broilers were inoculated with 0.1 mL of brain-heart infusion broth by the same route. The chicks were kept for seven days. They were observed at intervals of 6, 12 and 24 h post-inoculation during the first days. From the second day on, the chicks were observed at intervals of 12 h. According to the death time and to the scores of each lesion (aerosaculitis, pericarditis, perihepatitis, peritonitis and cellulitis), a formula to determine the Individual Pathogenicity Index was established. A value of 10 was established as the maximum pathogenicity rate for an inoculated bird. From this rate, 5 points corresponded to scores for gross lesions present at necropsy. For each lesion present, it represents 1 point. The remaining 5 points corresponded to the death time. To obtain the death time value, an index of 1, corresponding to the maximum value assigned to a death on the first day, was divided by the number of days that the birds were evaluated, resulting in a value of 0.1428, which corresponded to a survival bonus factor. It was possible to classify *E. coli* strains into four pathogenicity groups according to the pathogenicity index: high pathogenicity (pathogenicity index ranging from 7 to 10), intermediate pathogenicity (pathogenicity index ranging from 4 to 6.99), low pathogenicity (pathogenicity index ranging from 1 to 3.99) and apathogenic (pathogenicity index ranging from 0 to 0.99). The analysis of the strains according to their origin revealed that isolates from broiler bedding material presented a lower pathogenicity index.

Discussion: It is possible that the source of isolation implies in different results, depending on the criteria adopted. This data reinforces the importance of use a more accurate mathematical model to represents the biological phenomenon. In the study, all avian pathogenic *Escherichia coli* strains were classified based on a pathogenicity index and the concept of the death time represents an interesting parameter to measure the ability of the strain to promote acute and septicemic manifestation. The use of a support method for poultry veterinary diagnostic accompanying the fluctuation of the bacteria pathogenicity inside the farms may indicate a rational use of antimicrobial in poultry industry.

Keywords: APEC, colibacillosis, mathematical model, pathogenicity.

INTRODUCTION

Infections with avian pathogenic *Escherichia coli* (APEC) cause colibacillosis, an acute and systemic disease. Avian colibacillosis is a complex syndrome characterized by multiple organ lesions as aerosacculitis, polyserositis, septicemia and other mainly extraintestinal diseases in chickens, turkeys and other avian species. APECs are found in the intestinal microflora of healthy birds and in the environment. The infection of the respiratory tract by *E. coli* followed for septicemia is referred as an outstanding pathogen for the poultry industry, due to its several economic losses [1,3,6,7,20]. Environmental factors and the poultry immunological system influence the outcome of APEC infections. Thus, *in vivo* experiments are used to measure the ability of *E. coli* to be pathogenic in poultry [14-17]. One of the first published works on *in vivo* studies aimed to relate mortality rates in one-day-old chicks [4]. From this work on, other authors have proposed different criteria for results interpretation, although the methodology of the experiments is still similar and did not take into account the death time. Usually, the main parameter assessed by these tests concerns the mortality caused by each strain.

The aim of this study was to propose a new methodology for the classification of *E. coli* pathogenicity by the establishment of a pathogenicity index based in the lethality, death time and the ability of the strain to cause colibacillosis lesions in challenged animals.

MATERIALS AND METHODS

Experimental animals

A total of 2940 one-day-old broilers from commercial breeding hens were acquired from a Brazilian poultry company for the study. Birds were randomly assigned to groups containing 10 animals and housed in battery cages that were placed inside an isolation room with controlled temperature (32°C). Drinking water and commercial feed were provided *ad libitum*.

Escherichia coli *strains and inoculum preparation*

A total of 293 strains of *Escherichia coli* were randomly selected from our stock collection. These strains were isolated from three different broiler sources: cellulitis lesions (n = 162), broiler bedding material (n = 72) and respiratory diseases (n = 59). The strains were previously confirmed as *E. coli* through biochemical profile. The bacterial isolates were kept frozen at -20°C in brain heart infusion broth (BHI)[1] and glycerol[2]. An amount of 100 μL of the culture stocks were retrieved and cultured in BHI overnight at 37°C to obtain a final concentration of 10^9 UFC/mL. After this period, the inoculum was prepared in aliquots of 0.1 mL from the cultured BHI.

Inoculation

Groups of 10 one-day old chicks were subcutaneously inoculated in the abdominal region with 0.1 mL of the standard inoculum solution containing each of the 293 *E. coli* strains. A control group of 10 broilers were inoculated with 0.1 mL of BHI by the same route.

Mortality, evaluation of macroscopic lesions and bacterial reisolation

The chicks were observed at intervals of 6, 12 and 24 h post-inoculation during the first day. From the second until the seventh day, the chicks were observed at intervals of 12 h. Dead birds were collected for necropsy and for macroscopic evaluation of the presence of the following lesions: pericarditis, perihepatitis, peritonitis, airsacculitis and cellulitis. Chicks that survived until the seventh day were euthanized and necropsied. Animals that died on the first day and that had no lesions were submitted to bacterial isolation from the liver. The material was inoculated in BHI and incubated at 37°C for 24 h and then streaked in eosin methylene blue[1] and incubated at 37°C for 24 h to observe typical metallic green colonies of *E. coli*.

Establishment of Individual Pathogenicity Index

The Individual Pathogenicity Index (IPI) corresponds to the pathogenicity index for each bird in each group inoculated with a strain of *E. coli*. A value of 10 was established as the maximum pathogenicity rate for an inoculated bird. From this rate, 5 points corresponded to scores for gross lesions present at necropsy. For each lesion present, it represents 1 point. The remaining 5 points corresponded to the death time. To obtain the death time value, an index of 1, corresponding to the maximum value assigned to a death on the first day, was divided by the number of days that the birds were evaluated, resulting in a value of 0.1428, which corresponded to a survival bonus factor. Thus, every day that the birds survived was discounted 0.1428 of the death time value. Animals which died on the first day post inoculation and that had positive result on bacterial isolation were presumably died by

septicemia. Therefore, they received a score 1 for the death time value. According to the death time value and to the scores of each lesion, a formula to determine the IPI was established:

$$IPI = (Td \times 5) + Pc + Ph + Pt + A + C$$

Legend: Individual Pathogenicity Index (IPI), death time (Td), pericarditis (Pc), perihepatitis (Ph), peritonitis (Pt), aerosaculitis (A) and cellulitis (C).

Table 1 shows an example of the establishment of the individual pathogenicity index of a strain inoculated.

Table 1. Example of the establishment of the individual pathogenicity index (IPI) of *E. coli* inoculated in 10 one-day old chicks.

Strain	Chick	Td	SBF	A	Pc	Ph	Pt	C	IPI
1	1	1	1	1	1	1	1	0	10
1	2	2	0.8572	0	0	1	1	1	7.29
1	3	2	0.8572	1	1	1	1	1	9.29
1	4	3	0.7144	1	1	1	1	1	8.57
1	5	4	0.5716	0	1	1	1	1	6.86
1	6	4	0.5716	0	0	0	0	0	2.86
1	7	E	0	0	0	0	0	0	0
1	8	E	0	0	0	0	0	1	1
1	9	E	0	0	0	0	0	1	1
1	10	E	0	0	0	0	0	1	1

Time of death (Td); survival bonus factor (SBF); aerosaculitis (A); pericarditis (Pc); perihepatitis (Ph); peritonitis (Pt); cellulitis (C); Individual Pathogenicity Index (IPI); euthanized bird at the seventh day (E); lesion present (1); lesion absent (2).

The pathogenicity index for each inoculated strain was calculated according to the equation below:

$$PI = \frac{\sum (IPI)}{N}$$

Legend: pathogenicity index (PI), sum of individual pathogenicity index ($\sum$ (IPI)), total of inoculated chicks in the same group (N)

Statistical Analysis

Data were submitted to one-way analysis of variance and the means were compared by Kruskal-Wallis test, at a 1% significance level ($P < 0.001$) using SigmaStat software[3].

RESULTS

To determine the virulence of the strains after *in vivo* inoculation, we calculated the death time and we also analyzed the presence of five colibacillosis lesions. There was a significant difference ($P < 0.001$) in the death time among the strains isolated from the

three sources. Table 2 describes the death time mean according to the source of isolation.

In the present study, it was possible classify the *E. coli* strains in pathogenicity index as showed in Graphic 1. There was a predominance of strains with lower indexes (0 and 1), and a relatively homogeneous distribution among the PIs from 2 to 10.

Escherichia coli strains were classified into four pathogenicity groups according to the pathogenicity index: high pathogenicity (pathogenicity index ranging from 7 to 10), intermediate pathogenicity (pathogenicity index ranging from 4 to 6.99), low pathogenicity (pathogenicity index ranging from 1 to 3.99), apathogenic (pathogenicity index ranging from 0 to 0.99). There is significant difference ($P < 0.001$) among the pathogenicity index mean, as showed in Table 3.

The analysis of results according to the source of isolation, as described in Table 4, revealed significant difference ($P < 0.001$) in their pathogenicity index mean.

Table 2. Death time according to the source of isolation of *E. coli* strains.

Source of isolation	Total of strains	Death time mean (days)
respiratory diseases	61	1.43[a]
cellulitis	144	4.0[b]
broiler bedding material	59	5.7[b]

[a,b]Different letters in the same column represent significant difference ($P < 0.001$).

Table 3. Classification of 293 *Escherichia coli* strains in four categories of pathogenicity and their pathogenicity index mean.

| Classification	Total of strains	Pathogenicity Index (PI) mean
High Pathogenicity (H)	97	8.159[a]
Intermediate Pathogenicity (I)	59	4.571[b]
Low Pathogenicity (L)	103	1.685[c]
Apathogenic (A)	41	0.30[d]

[a,b,c,d]Different letters in the same column represent significant difference ($P < 0.001$).

Table 4. Pathogenicity index means and coefficient of variation of *E. coli* strains according to their source of isolation.

Source of isolation	Total of strains	Pathogenicity Index (mean ± standard deviation)	Coefficient of Variation
respiratory diseases	59	5.420 ± 2.991[a]	55.16
cellutis	162	4.622 ± 3.245[a]	70.21
broiler litter	72	2.319 ± 2.292[b]	98.83

[a,b]Different letters in the same column represent significant difference ($P < 0.001$).

Graphic 1. Distribution of the 293 *Escherichia coli* strains according to the Pathogenicity Index.

4
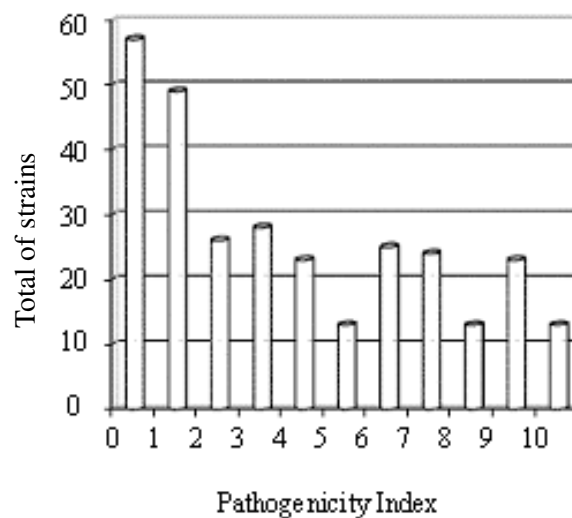
DISCUSSION

The concept of the death time represents an interesting parameter to measure the ability of the strain to promote acute and septicemic manifestation. Strains isolated of respiratory diseases presented a higher death time mean, reflecting the pathogenic behavior of these strains in the field. Therefore, it is possible that the source of isolation implies in different results, depending on the criteria adopted. This data reinforces the importance of survival bonus factor to use a more accurate mathematical model to represents the biological phenomenon.

The classification in four categories (high pathogenicity, intermediate pathogenicity, low pathogenicity, apathogenic) represents an advancement in *E. coli* studies. It is possible to observe that *E. coli* isolated from broiler bedding material presented a lower pathogenicity index, and the index was significantly different ($P < 0.001$) of the others sources. Some isolates from cellulites and respiratory diseases showed lower pathogenicity index. Thus, it is likely that in the field, these isolates probably were associated with other factors that provide favorable conditions for bacterial growth and lesions development [1]. On the other hand, some strains isolated from broiler bedding material showed a higher pathogenicity index. Among all the factors contributing to the appearance of lesions, poultry environment is a determining factor and acts directly on changes in the bacterial DNA. The genome of *Escherichia coli* consists of a conserved part, the so-called core genome, which encodes essential cellular functions and of a flexible, strain-specific part [5]. Genes that belong to the flexible genome code for factors involved in bacterial fitness and adaptation to different environments [5]. It is state that acquisition or loss of genetic information is of great importance for the adaptive evolution of bacteria, which cause disease [9]. Furthermore, the simultaneous acquisition of many genes by horizontal transfer allows transmission of characteristics related to disease in a single step. Thus, these species include pathogenic variants as well as commensal bacteria adapted to different host organisms [19]. In *Escherichia coli*, various genetic elements encode for pathogenicity factors as well as factors, which increase the fitness of non-pathogenic bacteria [5]. There are approximately 10^6 CFU of *E. coli* per gram in avian excreta and 15% of the strains isolated belong to pathogenic serogroups [8]. Thus, the monitoring of pathogenic *E. coli* in the poultry farms is important and becomes unavoidable. The difficulty in distinguishing pathogenic and no pathogenic strains of *E. coli* after bacterial isolation is possibly related to the complexity of the interactions that exist among the virulence factors of *E. coli*, beyond the subjectivity of conventional parameters used to determine the pathogenicity [18].

CONCLUSION

According to the new methodology proposed in this study, it was possible to identify statistically significant differences among the pathogenesis index of strains. The use of the survival bonus factor and the classification of five lesions after the inoculation of chicks reflected the virulence of the strains adequately. Therefore, the use of a support method for poultry veterinary diagnostic accompanying the fluctuation of the bacteria pathogenicity inside the farms may indicate a rational use of antimicrobial in poultry industry.

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