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Transmission identification of *Escherichia coli* aerosol in chicken houses to their environments using ERIC-PCR

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In order to study *E. coli* aerosol spreading from chicken houses to their surrounding air, air samples, including indoor and outdoor air (upwind 10 and 50 m as well as downwind 10, 50, 100, 200 and 400 m away) of 5 chicken houses were collected using six-stage Andersen microbial samplers and Reuter-Centrifugal samplers (RCS). *E. coli* concentrations (CFU/m^3 air) collected from different sampling sites were calculated. *E. coli* strains from chicken feces samples were also isolated. Furthermore, the enterobacterial repetitive intergenic consensus (ERIC)-PCR method was applied to amplify the isolated *E. coli* strain DNA samples. Through the genetic similarity analyses of the *E. coli* obtained from different sampling sites, the spreading of bioaerosol from animal houses to the ambient air was characterized. The results showed that the isolated *E. coli* concentrations in indoor air (9—63 CFU/m^3) in 5 chicken houses were higher than those in upwind and downwind air, but there were no significant differences between the indoor and downwind sites 10 m away from all the 5 houses (P>0.05). The phylogenetic tree indicated that a part of the *E. coli* (34.1%) isolated from indoor air had 100% similarity with those isolated from feces, and that most of *E. coli* isolated (54.5%) from downwind at 10, 50, 100 or even 200 m had 100% similarity with those isolated from indoor air or feces too. But those isolated from upwind air had a lower similarity (73%—92%) with corresponding strains isolated from indoor air or feces. Our results suggested that some strains isolated from downwind air and indoor air originated in the chicken feces, but most of isolates obtained from upwind air samples did not come from the chicken feces or indoor air. Effective hygienic measures should be taken in animal farms to prevent or minimize downwind spreading of microorganism aerosol.

chicken house, spread of *E. coli* aerosol, ERIC-PCR, similarity identification, epidemiological significance

Microorganisms and their products in bioaerosol from animal houses can cause serious air pollution. They may also affect the health and the production capability of the animals[1] and induce prevalence of aerosol infectious diseases. The polluted air in livestock farms is often associated with the outbreak of the epidemic diseases and the environmental problems. Some airborne bacteria in animal houses, including pathogenic bacteria, selective pathogenic bacteria and nonpathogenic bacteria[2], can cause diseases not only to animals but also to human beings[3,4]. Even a very limited number of pathogenic microorganisms in the atmosphere can cause direct respiratory tract infection, especially in the down-respiratory tract[5].

It is known that many airborne pathogen microorganisms, including viruses and bacteria, can spread over a large area through the air[6,7]. Examples of the potential threat of airborne pathogenic spreading include the foot and mouth disease[8], severe acute respiratory syndrome (SARS)[9,10], pandemic measles in the pre-vaccination era[11], airborne anthrax in the USA in 2001[12], and the spread of *Klebsiella pneumonia*[13]. All those highly

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pathogenic viruses, including some pandemic pathogens, serve as timely reminders that airborne infectious diseases remain a serious threat to human health.

Bioaerosol disseminated from animal houses to their environments has been studied with an emphasis on total bacterium amount[14,15], pathogenic bacteria and antibiotic resistances of the bacteria in animal houses and their ambient air[16,17].

It is difficult to differentiate between two strains that have very close genetic relationship using traditional bacterial taxonomy. However, ERIC sequences are 126 bp long and appear to be restricted to transcribed regions of the genome in *Enterobacteriaceae* species, either in intergenic regions of polycistronic operons or in untranslated regions upstream or downstream of open reading frames. The ERIC sequences are highly conserved at the nucleotide sequence level but their chromosomal locations and numbers differ among species[18]. So the ERIC-PCR method can serve as a valuable and sensitive tool for genetic differentiation of *E. coli* isolated from different sites[19,20].

It is interesting to further identify the bioaerosol source around animal houses and their spreading ways to the ambient air. In this study, the concentrations of airborne *E. coli* cells at different locations in chicken houses were measured and the genetic diversity of *E. coli* isolates obtained from every chicken house was analyzed using the ERIC-PCR method[21]. The degree of similarity between isolated strains was assessed by construction of a dendrogram. Our results indicated that the airborne *E. coli* in indoor air of chicken houses could spread to their ambient air via air exchange.

1 Materials and methods

1.1 Animal houses studied

Five chicken houses investigated in Tai'an, Shandong, China, between April and August 2006 were located outside of the villages in the city suburb, where there were no buildings or tall plants around the chicken houses. Chicken house A, at the foot of a hill, had a distance of more than 3000 m to the nearest village. Chicken house B, C, D and E were located to the east of the villages with distances ranging from 500 to 1000 m from the nearest village and had the same geographical characteristics. As the air samplers were collected, the meteorological conditions were recorded simultaneously. A description of these chicken houses is given in Table 1.

1.2 Airborne *Escherichia coli*

Six-stage Andersen samplers[22] were used to collect airborne *E. coli* in the indoor air of the animal houses. The samplers were placed near the middle of the stable about 1 m above the ground. The RCS was used to collect airborne *E. coli* from the outdoor air at different sites of upwind (10 and 50 m away from the chicken houses) and downwind (10, 50, 100, 200 and 400 m away from the chicken houses). Airflow rates for the Andersen sampler and RCS were 28.3 and 40 L/min, respectively. The Andersen sampler was equipped with MacConkey agar No.3 (Oxoid) plates and operated for 1 to 8 min and the RCS was equipped with MacConkey agar strips and operated for 0.5 to 8 min according to the sanitation condition. At each sampling site, five air samples were collected. The exposed agar plates and agar strips were incubated at 37℃ for 48 h. Then all the colonies appearing in the plates and strips were screened based on their Gram reactions using the "KOH assay". Gram-negative colonies were subcultured in MacConkey agar and their species were then identified by using the API 20 E system (Bio Merieux, Marcy-I'Etoile, France). The number of colonies was counted and the positive-hole correction was applied[22]. Counts were expressed as CFU/m^3 air. All isolates were stored at –20℃ with 20% glycerine.

Table 1 Description of the five chicken houses

Chicken house	Chicken	Layout	Inside			Outside		
			T (℃)	RH (%)	WS (m/s)	T (℃)	RH (%)	WS (m/s)
A	6000	floor unit	26	40	0	21	50	1.0－3.0
B	2200	cage unit	26	34	0	29	50	1.0－3.1
C	3000	cage unit	31	44	0	35	36	1.5－3.0
D	3500	cage unit	31	60	0	32	75	0－1.5
E	4500	cage unit	30	70	0	31	65	0－2.0

RH, relative humidity; WS, wind speed.

1.3 *Escherichia coli* in feces

Fresh fecal samples from healthy broiler or layer were collected aseptically on 5 feedlots and the samples were immediately transported to the laboratory in ice-cooled containers and analyzed within 24 h of collection.

Approximately 1 g of feces was transferred to a sterilized glass homogenizer containing 9 mL of 0.9% sodium chloride solution. The diluted samples were spread onto the surface of eosin methylene blue (EMB) agar (Tianhe, Hangzhou, China) which were incubated at 37℃ for 18 to 20 h. Colonies with a metallic sheen on EMB agar were picked and streaked onto MacConkey agar NO.3 (Oxoid). After overnight incubation at 37℃, one or two typical pink colonies were selected from each MacConkey agar plate and the isolates were tested based on the above-mentioned method in section 2.2.

1.4 DNA extraction

All *E. coli* strains were grown in 5 mL of Luria-Bertani (LB) broth (Oxoid) with moderate shaking for 18 h at 37℃. Then, 1.5 mL LB broth was taken out and centrifuged at 10000×*g* for 2 min. The cell pellet was resuspended in 100 μL of sterile double distilled water and kept in boiling water for 10 min. The culture was then cooled down by putting on ice for 5 min. The mixture was centrifuged at 12000×*g* for 2 min. The supernatant was removed and stored at –20℃ and used as the DNA templates for PCR analysis later[23].

1.5 ERIC-PCR

The primers ERIC1 (3′-CACTTAGGGGTCCTCGAATGTA-5′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′)[21] were synthesized by TaKaRa (Dalian, China). The PCR reaction mixture (25 μL) contained 1.5 U Taq DNA polymerase (TaKaRa), 300 ng/μL of each primer, 0.875 mmol/μL of each dNTP, 1×reaction buffer and 1.5 mmol/L $MgCl_2$ (TaKaRa). PCR amplification was performed in a thermocycler (Eppendorf, Germany) as follows: an initial denaturation (95℃, 5 min) followed by 32 cycles of denaturation (94℃, 1 min), annealing (51℃, 1 min), and extension (72℃, 3 min) with a single final extension (72℃, 16 min). The reaction products were stored at 4℃ until they were electrophoresed in a 1.2%—1.5% agarose gel with DL2000 DNA markers (TaKaRa).

1.6 ERIC fingerprints analysis

ERIC fingerprints of amplified DNA fragments resolved by electrophoresis were recorded. The PCR product band patterns in the gel were evaluated based on the presence (coded 1) or absence (coded 0) of polymorphic fragments amplified with the ERIC primers. Cluster analysis was performed with software package of NTSYS-pc (Version 2.10) for taxonomy and multivariate analyses[24], using an unweighted pair-group method, arithmetic average (UPGMA). In addition, each isolate was considered as an operational taxonomic unit (OTU). In order to reduce the number of OTUs in the dendrogram and facilitate interpretation, isolates of ⩾90% similarity were treated as a single isolate[25].

1.7 Data analysis

Statistical analysis was carried out using the SPSS for windows (English version 13.0). The significant differences in airborne bacterial concentrations among different sampling locations in chicken houses were analyzed using Student's *t*-test.

2 Results

2.1 Concentrations of airborne *Escherichia coli* at the different sampling sites

Median concentrations of airborne *E. coli* measured by an Andersen sampler in the indoor air samples for the 5 different chicken houses were 37, 13, 14, 63 and 9 CFU/m^3 air. At the upwind sites 10 and 50 m away from the chicken houses the concentrations of airborne *E. coli* were 2 and 1 CFU/m^3 (for House A), 7 and 2 CFU/m^3 (for House D), respectively. The concentrations of airborne *E. coli* from downwind sites 10 m away from the 5 chicken houses ranged from 3 to 24 CFU/m^3. Those from downwind sites 50 m away from the houses ranged from 0 to 16 CFU/m^3. The *E. coli* concentrations at the downwind sites 100 and 200 m away from the houses were 3 and 1 CFU/m^3 (for House A), and 2 and 2 CFU/m^3 (for House D), respectively. No airborne *E. coli* was found from the downwind sites 400 m away from the 5 chicken houses (Table 2).

The difference in the airborne *E. coli* concentration between indoor and upwind sites (10 and 50 m away) was significant for both house A and D ($P<0.05$). There was no significant difference in the airborne *E. coli* concentrations between the indoor and downwind sites 10 m away from all the 5 chicken houses ($P>0.05$). A significant difference was found not only between the indoor and downwind sites 50 m away from houses C

Table 2 Concentrations of airborne *E. coli* in the five chicken houses (CFU/m^3 air) (n = 5)

Chicken house		UW 50 m	UW 10 m	Indoor	DW 10 m	DW 50 m	DW 100 m	DW 200 m	DW 400 m
A	Max.	7	11	134	49	24	11	3	0
	Min.	0	0	7	0	0	0	0	0
	Median	1	2	37	12	6	3	1	0
B	Max.	0	0	59	35	12	0	0	-
	Min.	0	0	3	0	0	0	0	-
	Median	0	0	13	8	2	0	0	-
C	Max.	0	0	71	12	0	0	0	-
	Min.	0	0	0	0	0	0	0	-
	Median	0	0	14	3	0	0	0	-
D	Max.	10	27	236	80	40	10	10	0
	Min.	0	0	11	0	0	0	0	0
	Median	2	7	63	24	16	2	2	0
E	Max.	0	0	35	18	0	0	0	0
	Min.	0	0	0	0	0	0	0	0
	Median	0	0	9	5	0	0	0	0

CFU, colony forming units; UW, upwind; DW, downwind; "-", air samples were not collected at this site.

and E but also between the indoor and downwind sites 100 and 200 m away from all the 5 chicken houses (P<0.05).

2.2 ERIC-PCR analysis

The electrophoretic profiles of the ERIC-PCR products were determined for the *E. coli* strains isolated from indoor air, outdoor air and feces. The data matrix based on the DNA fragments and the dendrogram using the NTSYS-pc software were constructed, grouping all the *E. coli* isolates obtained in this study into different clusters or branches based on the ERIC-PCR pattern similarity. Furthermore, The ERIC DNA fragments from the isolates obtained from the same animal house were amplified in one PCR reaction and loaded on the same gel for a better comparison.

As shown in the UPGMA dendrogram for chicken house A (Figure 1), 28 *E. coli* isolates were grouped into 2 main clusters. Feces-6 compared to Indoor-6, -7 and Downwind10m-1, had 100% similarity. Indoor-1 and -2 had 100% similarity with Downwind10m-3, Downwind50m-1 and Downwind200m-1. Additionally, Feces-9 also had 100% similarity with Downwind100m-1. But the isolates from upwind air had low similarity with corresponding strains isolated from indoor air or feces, ranging from 73% to 91%. The similarity between Feces-3 and Upwind10m-1, Feces-3 and Upwind50m-1/2 was 77% and 73%, respectively. However, Upwind50m-1, -2 and Downwind10m-2 shared 91% similarity, indicating that the 3 strains belonged to the single isolate[25].

Our results showed that Feces-2, -3 had 100% similarity with Indoor-1, -3 and Downwind50m-1 in House B (Figure 2). Feces-10, -11 also had 100% similarity with Indoor-5, Downwind10m-2, -3. But Downwind10m-1 had only 83% similarity with corresponding strains isolated from feces (e.g. Feces-2) or indoor air (e.g. Indoor-1).

In house C (Figure 3), the following strains shared 100% similarity: Feces-2 with Indoor-2; Feces-3 with Indoor-4 and Downwind10m-1; Feces-4, -6 and -7 with Indoor-1. However, Downwind10m-2 had a lower similarity (44%—80%) with all the strains isolated from the feces and indoor air.

In House D (Figure 4), 100% similarity was found among the following samples: Feces-8 with Downwind100m-2; Feces-10 with Indoor-11; Feces-11, -12 with Indoor-4; Indoor-1, -5 with Downwind10m-2. A similarity of 92% between Feces-9 and Upwind50m-2 indicated that the 2 strains belong to the single isolate too[25]. The same degree of similarity was also found in the following two pairs: Feces-3 and Downwind200m-1; Feces-6 and Downwind50m-3. Nevertheless, a lower similarity (79%) existed among Feces-10, -11, -12 and Upwind50m-1.

In House E (Figure 5), Feces-2, -4, -8 and Indoor-4 shared 100% similarity. The same results were also ob-

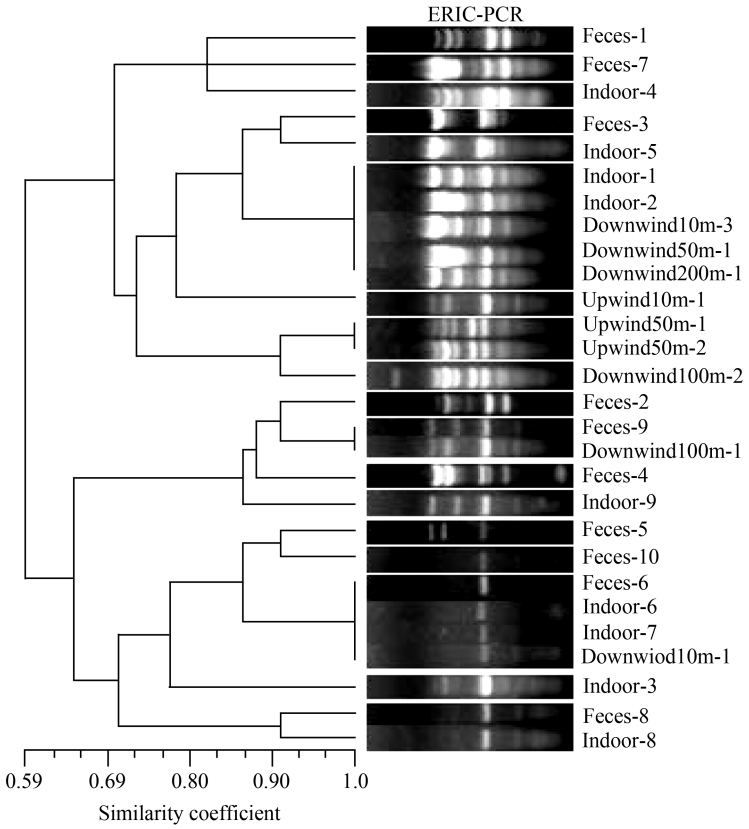

Figure 1 Dendrogram of *E. coli* strains in chicken house A based on ERIC-PCR analysis. Feces-1 means the first *E. coli* strain isolated from feces; Indoor-2 means the second *E. coli* strain isolated from indoor air; Upwind50m-1 means the first *E. coli* strain isolated from upwind at 50 m; Downwind100m-1 means the first *E. coli* strain isolated from downwind at 100 m.

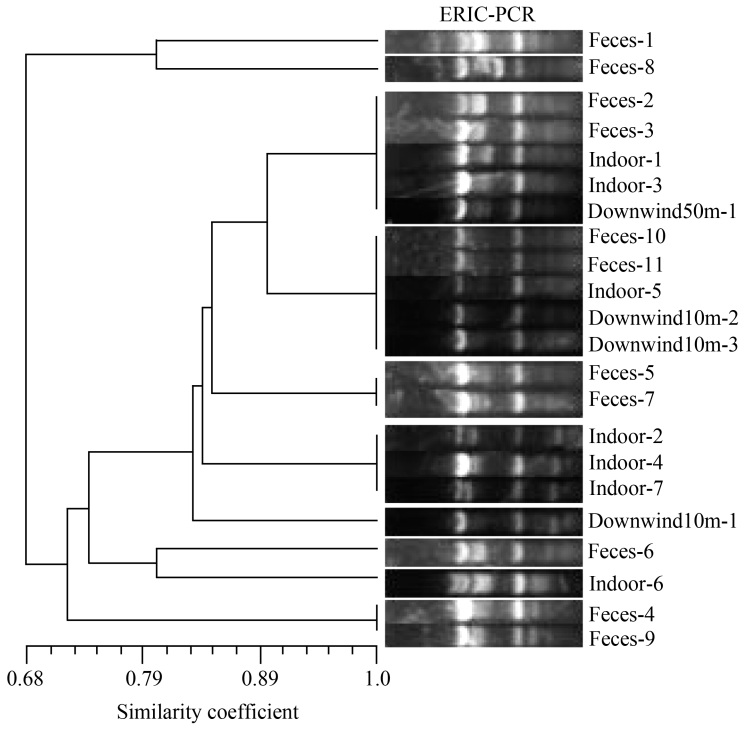

Figure 2 Dendrogram of *E. coli* strains in chicken house B based on ERIC-PCR analysis.

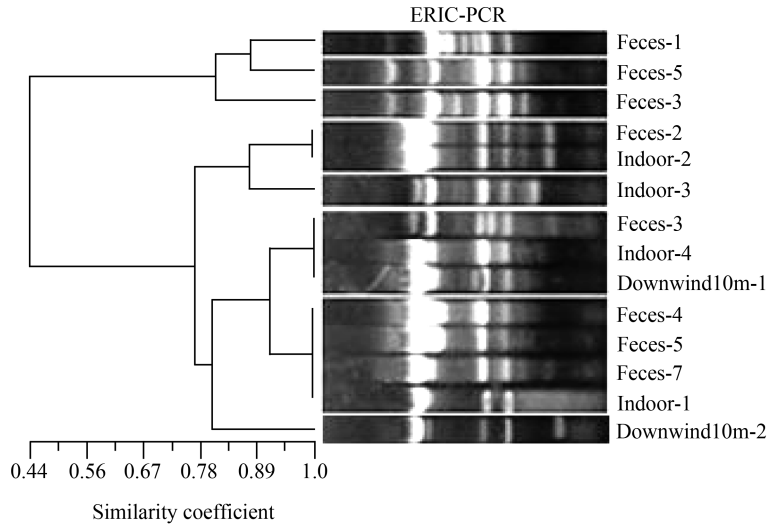

Figure 3 Dendrogram of *E. coli* strains in chicken house C based on ERIC-PCR analysis.

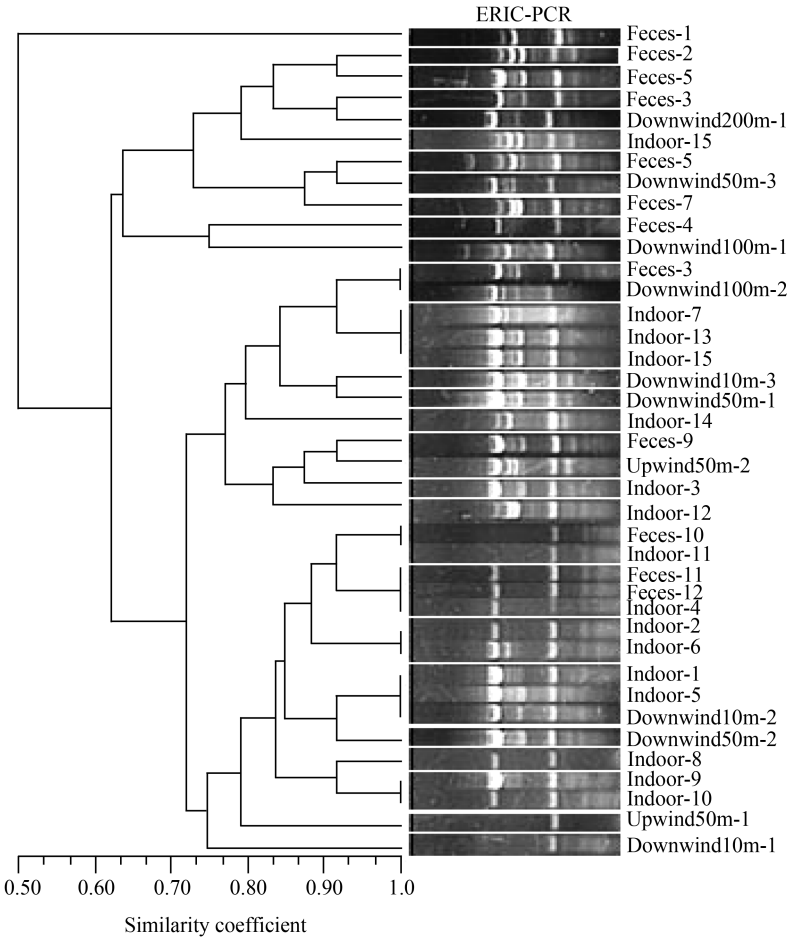

Figure 4 Dendrogram of *E. coli* strains in chicken house D based on ERIC-PCR analysis.

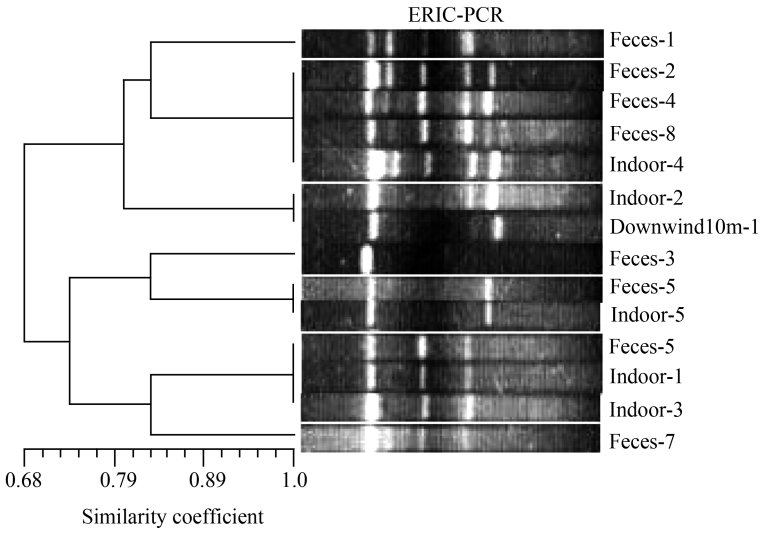

Figure 5 Dendrogram of *E. coli* strains in chicken house E based on ERIC-PCR analysis.

served when comparing the following samples: Feces-5 with Indoor-5; Feces-6 with Indoor-1 and -3; Indoor-2 with Downwind10m-1. Airborne *E. coli* was not isolated from upwind (10 and 50 m away from the house) or downwind sites (≥50 m away from the house).

3 Discussion

E. coli, a member of the family *Enterobacteriaceae*, is present as normal flora in the lower intestine of both human beings and animals[26]. Under special conditions, it can cause colibacilosis and be discharged with feces. Thereby, *E. coli* is often used as an indicator to trace the source of environmental, water and food pollution[27–29]. The ambient air of animal houses is often polluted with airborne *E. coli*[30,31]. Hojovec et al. (1977) evaluated the air quality in poultry house using *E. coli* as an indicator[32]. It is well known that out of many different types and strains of *E. coli*, a few are potentially pathogenic. Most enteropathogenic *E. coli* strains infect animals or human beings through the alimentary tract, contagions and respiratory system[33]. They cause illness by a variety of infective and toxin-producing mechanisms. In poultry, *E. coli* can cause many diseases such as septicemia, swollen head syndrome, omphalitis, cellulitis, yolk-sack infection and respiratory tract infections[34,35]. The resultant morbidity and mortality have led to serious economic losses to the poultry industry[1]. In human beings, it can cause many diseases such as hemolytic uremic syndrome (HUS), haemorrhagic colitis (HC), neonatal meningitis and bloody diarrhea[36,37].

In the present study, *E. coli* was used as an indicator to study possible air pollution caused by chickens because of a lot of concerns on the public hygiene and epidemiology related to these animal houses. Our results showed that the difference in the *E. coli* concentrations between indoor and upwind air (sampled at the distances of 10 and 50 m from the houses) was significant for the house A and D ($P<0.05$), and the same results were also found between indoor air and downwind air at the sites >50 m away from the houses. However, the difference in the *E. coli* concentrations among indoor air and downwind air sampled at the sites 10 and 50 m away from the houses A, B and D were not significant ($P>0.05$). All these results indicated that concentrations of *E. coli* aerosols in the houses were much high, and that it was spread from indoor to outdoor through air exchange, especially to the downwind sites (≤50 m). In addition, there was no significant difference ($P>0.05$) in the airborne *E. coli* concentrations among the different downwind sites (50, 100 and 200 m) of the houses B, C and E, indicating airborne *E. coli* could be spread far away (≥200 m) based on the meteorological conditions. Airborne *E. coli* was not collected in the upwind air at the sites 10 or 50 m away from the houses B, C or E and in downwind air at the sites 400 m away from the chicken houses A, D or E. It was probably because the following reasons: (1) the concentrations of airborne *E. coli* were very low in these sampling sites; (2) many environmental factors such as relative humidity, temperature, ultraviolet (UV) radiation, oxygen content, specific ions, various pollutants and air-associated factors influenced

the ability of microorganisms to survive in the air[38]; (3) the aerosolization of microorganisms and sampling stress could also lead to a loss of culture viability[39,40].

Bruijn (1992) found that the ERIC-PCR products analyzed on agarose gels were highly specific for each strain and ERIC-PCR patterns revealed their differences more clearly, with a preliminary analysis of over 30 *Rhizobium meliloti* isolates[41]. Judd (1993) found that the results obtained by restriction fragment length polymorphism hybridization analyses were correlated with the phylogenetic classification of *B. japonicum* serocluster 123 strains obtained using ERIC-PCR[42]. The ERIC-PCR has become a powerful tool for the molecular genetic analysis of bacteria and for bacterial taxonomy, for it allows the fingerprinting of individual genera, species, and strains and can help to determine phylogenetic relationships. It also can be used as an effective technique to trace the source pollution used in many studies[18,20,25,43].

In this experiment, the degree of similarity among the isolates was assessed as shown in the dendrogram tree, which also enabled the comparison of different clusters of the isolates collected from different sampling sites in each chicken house. The ERIC-PCR results showed that the *E. coli* isolates from different sites in 5 chicken houses exhibited a high degree of polymorphism in their DNA sequences (Figures 1－5). Out of 41, 14 strains (34.1%) isolated from indoor air samples in the 5 chicken houses (2/9, 3/7, 3/4, 2/16 and 4/5 in the house A, B, C, D and E, respectively) had 100% similarity with the strains isolated from feces in all these chicken houses, indicating that the same strains were isolated from the indoor air and feces, namely the fecal strains could be aerosolized and spread to the indoor air in chicken houses. But some *E. coli* isolated from indoor air have the far genetic distance (< 90% similarity) with corresponding strains isolated from feces. For example, in the house A the similarity between Indoor-4 and Feces-1 or Feces-7 was 82%; in the house B Indoor-6 and Feces-6 shared 80% similarity. This observation might result from the following reasons: (1) some strains isolated from the indoor air might come from other sources (e.g. feed, water, etc.); (2) these strains might originate from the feces but our fecal sampling failed to isolate these strains.

Similarly, 12 strains out of 22 in total (54.5%) (5/6, 3/4, 1/2, 2/9 and 1/1 in the house A, B, C, D and E, respectively) isolated from the air of the downwind sites had 100% similarity with the strains isolated from feces or indoor air in 5 chicken houses, indicating that the strains in the feces or indoor air could spread to the outdoor air via air exchange. However, some strains isolated from downwind air had < 90% similarity with the strains isolated from feces or indoor air in 5 chicken houses. For instance, Downwind10m-1 had only 83% similarity with strain Feces-2 or Indoor-1 in house B; Downwind10m-2 had ≤80% similarity with all strains isolated from feces (e.g. Feces-3) or indoor air (e.g. Indoor-1) in house C, etc. These results indicated that some strains isolated from downwind sites did not originate from the stables. It is obvious that outdoor air can be easily and quickly affected by anthropogenic activities[44] and other environmental factors.

Our results also showed low similarity between some airborne *E. coli* strains at the upwind sites and the ones in the feces in the house A and D, indicating that some strains in the upwind air in the two chicken houses did not come from the chicken feces or indoor air. Nevertheless, around house A, the strains Upwind50m-1 and -2 had 91% similarity with the one of Downwind10m-2, suggesting that the upwind airborne bacteria might spread to the downwind air. And the isolates Feces-9 and Upwind50m-2 had 92% similarity in the chicken house D, indicating that the 2 strains can be considered as the same isolate[25], namely, outdoor air and indoor air could exchange each other.

Taken together, by using the accurate ERIC-PCR technology *E. coli* in feces was found aerosolized and spread to outdoor air, especially to downwind air of the chicken houses via air exchange. This process, assumably affected by local micro-climate, can cause the ambient air outside of the animal houses polluted and further threaten the neighboring inhabitants and the chickens themselves.

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