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SUMMARY. This study was undertaken to observe the prevalence, serogroup, avian pathogenic *Escherichia coli* (APEC)-associated virulence gene, randomly amplified polymorphic DNA (RAPD) pattern, and antibiotic resistance genes of *E. coli* in backyard layers and their environment in India. From the 360 samples of healthy layers and their environment, 272 (75.5%) *E. coli* were isolated. The majority (28.67%) of them were untypeable. Among the studied virulence genes (*papC*, *tsh*, *iucC*, *astA*), 52 (14.32%) isolates were found to possess *astA*, including the isolates from the drinking water of the birds (4/272, 1.47%). These strains belonged to 18 different serogroups. Most of the isolates were typeable by RAPD and they produced different patterns. Phenotypic resistance of the isolates was most frequently observed to erythromycin (95.83%), chloramphenicol (87.52%), and cotrimoxazole (78.26%). None of the isolates was found to possess extended-spectrum beta-lactamases (bla_{TEM}, bla_{SHV}, bla_{CTX-M}) or quinolone resistance (*qnrA*) genes by PCR. The present study was the first attempt in India to assess APEC distribution in backyard poultry production.

RESUMEN. Repertorio de virulencia, caracterización y análisis de los patrones de resistencia a los antibióticos de *Escherichia coli* aisladas de gallinas de postura de traspatio y de su medio ambiente en la India.

Este estudio se realizó para determinar la prevalencia, el serogrupo, los genes asociados a virulencia de *Escherichia coli* patógena para las aves (APEC), los patrones de ADN polimórfico amplificado aleatoriamente (RAPD), y los genes de resistencia a los antibióticos de *E. coli* en gallinas de traspatio y su ambiente en la India. De las 360 muestras de gallinas sanas y de su ambiente, se aislaron 272 cepas de *E. coli* (75.5%). La mayoría (28.67%) de ellas no fueron tipificables. Entre los genes de virulencia estudiados (*papC, tsh, iucC, astA*), se encontró que 52 aislamientos (14.32%) poseían el gene *astA*, incluyendo los aislamientos de agua de bebida de las aves (4/272, 1.47%). Estas cepas pertenecían a 18 serogrupos diferentes. La mayoría de los aislamientos fueron tipificables mediante RAPD y produjeron diferentes patrones. La resistencia fenotípica de los aislamientos se observó con mayor frecuencia contra eritromicina (95.83%), cloranfenicol (87.52%), y contra cotrimoxazol (78.26%). Se encontró mediante PCR que ninguno de los aislamientos poseía genes de un espectro extendido de beta-lactamasas (bla_{TEM}, bla_{SHV}, bla_{CTX-M}), o resistencia contra quinolonas (*qnrA*). El presente estudio es el primer intento en la India para evaluar la distribución de *E. coli* patógena para la producción de aves de traspatio.

Key words: APEC, backyard layer, ESBL, India, PCR, RAPD, West Bengal

Abbreviations: APEC = avian pathogenic *Escherichia coli*; CLSI = Clinical and Laboratory Standards Institute; EMB = eosin methylene blue; ESBL = extended-spectrum beta-lactamase; RAPD = randomly amplified polymorphic DNA; RIR = Rhode Island red; UPEC = uropathogenic *Escherichia coli*; UT = untypeable

Escherichia coli is present as commensal microflora of the intestinal tract of poultry birds and their environment but certain strains among them, known as avian pathogenic *Escherichia coli* (APEC), are able to cause colibacillosis due to possession of specific virulence factors. Avian colibacillosis is characterized by a diverse array of lesions. The infection initiates with septicemia which is followed by either sudden death or localized inflammation in vital organs causing perihepatitis, airsacculitis, pericarditis, salpingitis, omphalitis, osteomyelitis, and so forth (14). The disease is distributed worldwide and has major economic impact, especially in the broiler industry due to morbidity, lack of uniformity in a flock, lowered production, increased condemnation at the slaughter plant, and mortality (1). Currently there is a resurgence of this infection among layer chickens as observed in Europe (22,43) and Asia (20), including India (23). The layers kept in backyard farming also possess multidrug-resistant *E. coli* as reported in Costa Rica (21) as well as in India (9).

APEC possesses several virulence factors such as colicin V (cvaC), siderophore receptor (iroN), increased serum survival gene (*iss*), outer membrane protein (traT), and hemolysin (hlyD) (37). Among the virulence factors identified four are described as major factors, such as P fimbriae (*papC*), which helps to adhere to chicken internal organs, temperature-sensitive hemagglutinin (*tsh*), which plays a role

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in colonization of the air sacs, the aerobactin iron sequestering system (*iucC*), and enteroaggregative toxin (*astA*) (16,41). These virulence factors (*iucC*, *tsh*, *papC*) help the pathogen to establish extraintestinal infection in birds as well as in humans also because both the APEC and uropathogenic *E. coli* (UPEC) can produce extraintestinal infection with the help of some common virulence factors (15,36).

Transmission of antimicrobial-resistant *E. coli* in the human food chain from the chicken is a well-recognized phenomenon (19). Extended-spectrum beta-lactamases (ESBL), produced by *E. coli*, result in resistance against later-generated cephalosporins (except cephamycins and carbapenems) and monobactam. Currently, ESBL-producing organisms are becoming a major threat for patients in the hospital and community (17). The major ESBL (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$) and quinolone resistance genes (*qnrA*), if possessed by APEC, have the potential to be transferred to humans through the food chain and may complicate extraintestinal infections such as urinary tract infections in humans.

The present study was undertaken in West Bengal, a major egg-producing state in India, where a majority of the production is offered from the layers kept in a backyard farming system. The objective of this study was to examine *E. coli* isolates from backyard layers and their environment from different agroclimatic zones for the presence of serogroups, virulence genes associated with APEC capable of establishing extraintestinal infection in humans, and major antibiotic resistance genes as well as to perform randomly amplified polymorphic DNA (RAPD)-based characterization of the isolates.

MATERIALS AND METHODS

Sampling. Three-hundred and sixty samples ($n = 360$) for bacteriologic analysis from the birds kept in backyard systems, their environment, and eggs were collected from four agroclimatic zones of West Bengal, India (Terai, Red Laterite, New Alluvial, Coastal) during July–September 2011. The collection area included Jalpaiguri and Dinajpur districts (Terai zone, $n = 90$), Howrah District (New Alluvial zone, $n = 90$), West Midnapur District (Red Laterite zone, $n = 90$), and South 24 Parganas District (Coastal zone, $n = 90$). The average minimum–maximum temperature and rainfall was 22–31 C and 8 mm in the Terai zone, 27–34 C and 5 mm in Red Laterite zone, and 26–33 C and 7 mm in New Alluvial and Coastal zones, respectively, during the sample collection period. The samples were comprised of cloacal swabs of the birds ($n = 10$), their feed (4 g, $n = 10$), drinking water (10 ml, $n = 10$), utensil swabs ($n = 10$), litter (4 g, $n = 10$), swabs from the walls of the poultry house ($n = 10$), dried manure under the house (4 g, $n = 10$), soil (4 g, $n = 10$), and the eggs ($n = 10$), which were collected randomly. The sample size and amount of sample was determined per earlier reports (25,34). The birds belonged to the Rhode Island red (RIR) breed and were of all age groups (7 days–3 mo) and either sex and were apparently healthy. The age of the birds was divided into three groups; group 1 (1–4 wk), group 2 (5–8 wk), and group 3 (>8 wk). The average flock size maintained by the farmers was 20–25. The farmers offered boiled rice, raw vegetable waste, and seeds as feed and locally available water for the birds to drink. In most cases the drinking water was not regularly changed in the troughs. The birds generally roamed around the farmer's house in the daytime and took shelter in bamboo-made poultry houses at night. The birds were called for feed and drinking water at a specific time by the women of the farms.

The cloacal samples and swabs from the walls of the poultry houses and utensils were collected with sterile swabs (HiMedia, Mumbai, India) whereas, feed, litter, dried manure under the house, and soil samples were collected in sterile collection vials (HiMedia). The procured eggs were kept in sterile plastic zipper bags in an icebox with an ice pack. All the samples were transported to the laboratory in a thermoflask with an ice pack.

Processing of samples for bacteriologic analysis. All the swabs (cloacal and utensil) were directly inoculated into nutrient broth (HiMedia) for bacterial growth. The feed, litter, dried manure, and soil samples were also directly inoculated into nutrient broth at 4 g of sample in 36 ml of broth. The drinking water samples were inoculated into nutrient broth at 5 ml of sample in 45 ml of broth. The egg yolks and white portions were aseptically taken and were homogenized and mixed with 200 ml of buffered peptone water and incubated at 37 C (32).

Isolation of *E. coli* from collected samples. The growth in nutrient broth was transferred to MacConkey's agar (HiMedia) and again incubated at 37 C overnight. The next day, 2–3 rose-pink colonies were randomly picked and transferred to eosin methylene blue (EMB) agar (HiMedia) followed by an overnight incubation at 37 C. The colonies were observed for metallic sheen and a single colony was streaked into a nutrient agar slant for further biochemical confirmation. All pure cultures obtained from the nutrient agar slant were subjected to Gram staining and standard biochemical tests as described earlier (45). The strains were stored in glycerol broth at −70 C for subsequent characterization.

Serogrouping. All the *E. coli* isolates, after confirmation by biochemical tests, were sent for O-serogrouping to the National *Salmonella* and *Escherichia* Center, Central Research Institute, Kasuli, Himachal Pradesh, India.

Bacterial DNA extraction. For PCR-based detection of APEC virulence and major ESBL or *qnrA* genes from all the *E. coli* isolates, DNA was extracted as per the previously described method (44).

PCR-based detection of characteristic APEC virulence genes. The virulence genes for APEC (*papC*, *tsh*, *astA*, *iucC*) isolates were confirmed by PCR using the primer sequences and cycle condition of earlier works (16,41). All the reagents were procured from Genetix Biotechnology Asia Pvt. Ltd. (New Delhi, India). The amplification was conducted in a thermocycler (Eppendorf, Hamburg, Germany). The amplified product was visualized by a gel documentation system (UVP, Cambridge, United Kingdom) after electrophoresis in 2% (w/v) agarose gel containing ethidium bromide (0.5 μg/ml; SRL Laboratories, Mumbai, India) (38).

Nucleotide sequencing of selected PCR products of the virulence genes. The selected PCR products were sequenced on both strands in an ABI 3730 XL automated sequencer (Applied Biosystems, Foster City, CA). The sequence homology searches were conducted using the Basic Local Alignment Search Tool algorithm (www.ncbi.nlm.nih.gov/BLAST).

Molecular characterization of *E. coli* isolates by RAPD-PCR. The molecular characterization of all the *E. coli* isolates was done by RAPD-PCR using a single primer 6 (Genetix Biotechnology Asia Pvt. Ltd.) in a thermocycler (Eppendorf) per the protocol of Chansiripornchai *et al.* (6). The PCR products were then electrophoresed in 2% (w/v) agarose gel containing ethidium bromide (0.5 μg/ml) (38).

Antibiotic resistance of *E. coli* isolates. All the PCR-confirmed *astA* genes possessing *E. coli* isolates were tested for their sensitivity and resistance to different antibiotics by the disc diffusion method. The minimum inhibitory concentration breakpoints were used to interpret the zone diameter for each antibiotic as mentioned in Clinical and Laboratory Standards Institute (CLSI) guidelines (8). The antibiotic discs used were ampicillin (10 μg), amikacin (30 μg), cefotaxime (30 μg), cephalexin (30 μg), ceftriaxone (30 μg), ceftizoxime (30 μg), cephalothin (30 μg), cephaloridine (10 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), cotrimoxazole (25 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), levofloxacin (5 μg), neomycin (30 μg), norfloxacin (10 μg), streptomycin (10 μg), and tetracycline (30 μg). All were obtained from HiMedia. The discs with CLSI-recommended antibiotic concentration were used. However, if not available, the discs (e.g., cotrimoxazole, cephalexin) with the concentration offered by the manufacturer were used.

PCR-based detection of major ESBL (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$) and quinolone resistance genes (*qnrA*). All the *E. coli* isolates, including controls, were subjected to PCR for detection of bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and *qnrA* genes using the primers and the cycle conditions described earlier (4,46,47).

Table 1. Isolation of *Escherichia coli* from backyard layers and their environment in different agroclimatic zones of West Bengal, India.

Agro-climatic zone	Samples from which *E. coli* was isolated	*n*	*E. coli* (%)
Terai	Cloacal swab, feed, drinking water, litter, soil	90	83 (92)
New Alluvial	Cloacal swab, feed, drinking water, litter	90	31 (35)
Red Laterite	Cloacal swab, feed, drinking water, litter	90	88 (98)
Coastal	Cloacal swab, feed, drinking water, litter	90	70 (78)
Total		360	272 (75.5)

Statistical analysis. Univariate analysis of variance for detection of significant differences between isolate numbers of *E. coli*, with the age group of the birds and their agroclimatic zone, was performed in SPSS version 21 (SPSS Inc., Chicago, IL). Further, the association of the antibiotic resistance among the *E. coli* isolates, with the source of isolation, serogroups, and RAPD pattern, was also calculated by a chi-square test using SPSS version 21 (SPSS Inc.).

RESULTS

Isolation of *E. coli* from collected samples. From the 360 cloacal swabs of apparently healthy layers, and their environmental samples (feed, drinking water, litter, soil) examined, a total of 272 isolates (75.5%, Table 1) were identified as *E. coli* on the basis of characteristic pink-colored colonies on MacConkey agar, a metallic sheen in EMB agar, and the gram-negative small rod appearance in stained smears; biochemically they were catalase (+ve), oxidase (−ve), indole (+ve), methyl-red (+ve), Voges-Proskauer (−ve), citrate (−ve), and urease (−ve), characteristic of the standard *E. coli* strain. All the *E. coli* were isolated from birds of the lower age group (1–4 wk, age group 1). A statistically significant difference was observed in the number of *E. coli* isolates between age group 1 (1–4 wk) and age group 2 (4–8 wk) at $P < 0.01$ and between age group 1 (1–4 wk) and age group 3 (>8 wk) at $P < 0.01$. The highest *E. coli* isolation rate in birds (cloacal swab) was observed in the Terai zone (20/272; 7.3%) followed by Red Laterite (13/272; 4.7%), Coastal (12/272; 4.4%), and the New Alluvial zone (10/272; 3.6%), which was statistically significant at $P < 0.05$. No *E. coli* were isolated from environmental samples (except feed, drinking water, litter, and soil) or egg samples collected (Table 1).

Serogrouping. Among the 272 *E. coli* isolates, 78 strains (28.67%) were untypeable (UT) and the remaining isolates (194) were distributed among 47 different O-serogroups. The serogroups isolated from the birds with higher frequency were UT, O84, O147, and O60 whereas the serogroups isolated from the feed, drinking water, litter, and soil were UT, O106, O123, and O170, respectively. The APEC serogroups such as O1, O2, O8, O22, and O78 were also isolated from the studied clinically healthy birds kept in all of the four agroclimatic zones. Among them, O8 was isolated with the highest frequency (16/272, 5.88%) from the birds, especially from those kept in the Terai zone.

Detection of characteristic APEC virulence genes in *E. coli* isolates. *Escherichia coli* harboring a single APEC virulence gene (*astA*) was detected in 52 (14.32%) of the 272 *E. coli* isolates. The sequence of the selected PCR product (*astA*) was found to be in consensus (98%) with the *astA* gene of *E. coli* isolated from a different country (GenBank accession numbers HM099899, HM099895, M099894). Other studied virulence genes (*papC, tsh, iucC*) were not detected in any of the *E. coli* isolates. The present study also detected the *astA* gene in *E. coli* isolates (4/272, 1.47%) from the drinking water of birds in the Coastal zone. The *E. coli* strains harboring the *astA* gene only belonged to 18 different serogroups (O2, O8, O10, O55, O74, O76, O78, O84, O90, O91, O101, O106, O123, O138, O143, O147, O170, O172) whereas 16 and 4 strains were UT and rough, respectively (Table 2).

RAPD analysis of *E. coli* isolates. All 272 *E. coli* strains were characterized by RAPD-PCR to determine the genetic diversity among the strains. The majority of the strains ($n = 212$) were typeable with primer 6 and they produced 20 different patterns. However, no zone- or serogroup-related specific pattern was observed whereas all the *astA* gene-possessing *E. coli* produced four (A, B, C, D) distinct patterns which were correlated statistically with antibiotic resistance (Table 2).

Antibiotic resistance of *E. coli* isolates. Resistance of *E. coli* isolates was observed most frequently to erythromycin (95.83%), chloramphenicol (87.52%), cotrimoxazole (78.26%), gentamicin (65.23%), and tetracycline (42.76%). No resistance was observed against cefotaxime, cephalexin, ceftriaxone, ceftizoxime, cephalothin, cephaloridine, ciprofloxacin, and levofloxacin. However, intermediate reaction was observed against ampicillin, amikacin, neomycin, norfloxacin, and streptomycin. Further, none of the isolates was found to possess ESBL (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$) or quinolone resistance genes (*qnrA*) in PCR.

A statistically significant correlation was detected between the antibiotic resistance, the RAPD pattern (of *astA* gene-possessing *E. coli*), and the zone of isolation of *E. coli* (Table 2). Due to a limited number of categorical isolates, it was not possible to draw any conclusion with respect to serogroups. The tetracycline resistance was more likely to occur among the *E. coli* isolates with RAPD pattern A (58%) than with the isolates possessing other patterns. Similarly, gentamicin resistance was more frequently associated with isolates with pattern B (55.5%). However, for both tetracycline ($P = 0.06$) and gentamicin ($P = 0.058$) the difference is marginally significant. Furthermore, the resistance against cotrimoxazole, erythromycin, gentamicin, and tetracycline among the *E. coli* isolates was found to have a statistical correlation with the agroclimatic zone of isolation. The Terai zone was significantly more frequently associated with the resistance against gentamicin ($P = 0.001$), tetracycline ($P = 0.007$), and cotrimoxazole ($P = 0.04$) whereas the New Alluvial zone was significantly more frequently associated with tetracycline ($P = 0.007$) and cotrimoxazole ($P = 0.04$). However, the erythromycin resistance was significantly ($P = 0.003$) higher when observed in isolates from the entire four zones.

DISCUSSION

APEC is found to be responsible for avian collibacillosis, an extraintestinal syndrome commonly encountered in broiler birds and which has a major economic impact worldwide. Currently layers in different parts of the world, including India, are also affected with APEC. However, systematic molecular epidemiologic study on APEC infection in layer birds kept in a backyard system is scarce. Earlier we reported the occurrence of multidrug-resistant *E. coli* in backyard layer birds in West Bengal, India (9). The present study was aimed to detect the serogroups, APEC-associated virulence genes

Table 2. Characterization of *Escherichia coli* isolated from backyard layers and their environment in West Bengal, India.

Agro-climatic zone	Serogroup	Virulence genotype				RAPD[A] profile	Antibiotic[B] resistance
		papC	*tsh*	*astA*	*iucC*		
Terai (cloacal swabs)	O8 (2)	−	−	+	−	A	E, C, Co, G
	O8 (2)	−	−	+	−	B	E, C, Co, G
	O76 (2)	−	−	+	−	D	E, C, Co, G, T
	O 138	−	−	+	−	ND	E, C, G, T
	O143	−	−	+	−	A	E, Co, G, T
	UT (3)	−	−	+	−	A	E, C, Co, G, T
	UT (2)	−	−	+	−	C	E, C, Co, G, T
New Alluvial (cloacal swabs)	O2	−	−	+	−	B	E, C, Co, G
	O55	−	−	+	−	D	E, C, Co, G
	O106	−	−	+	−	C	E, C, Co
	UT (5)	−	−	+	−	A	E, C, Co, G, T
Red Laterite (cloacal swabs)	O8	−	−	+	−	B	E, C, Co, G
	O10	−	−	+	−	C	E, C, G
	O84 (3)	−	−	+	−	ND	E, C, Co, T
	O84	−	−	+	−	A	E, C, Co
	O90	−	−	+	−	A	E, G
	O91	−	−	+	−	C	E, C
	O147 (2)	−	−	+	−	D	E, C, G, T
	Rough	−	−	+	−	A	E, C, Co
	UT (2)	−	−	+	−	D	E, C, Co, G
Coastal (cloacal swabs)	O74	−	−	+	−	A	E
	O78 (3)	−	−	+	−	C	E, C, Co, G
	O91	−	−	+	−	D	E, C
	O101 (2)	−	−	+	−	ND	E, Co, G
	O172	−	−	+	−	B	E, Co
	Rough (3)	−	−	+	−	ND	E, C, Co
	UT	−	−	+	−	A	E, C, Co, G
Coastal (drinking water)	O106	−	−	+	−	A	T
	O123	−	−	+	−	ND	C, T
	O170	−	−	+	−	ND	C, T
	UT(3)	−	−	+	−	D	E, C, Co, G

[A]ND = not detected.
[B]E = erythromycin, C = chloramphenicol, Co = cotrimoxazole, G = gentamicin, T = tetracycline.

capable of establishing extraintestinal infection in humans, antibiotic resistance genes, and the RAPD pattern of *E. coli* isolates from backyard layers and from their environment in different agroclimatic zones (Terai, New Alluvial, Red Laterite, and Coastal) of West Bengal, India.

The present study detected a high prevalence (75%) of *E. coli* in healthy backyard layers and from their environment within four agroclimatic zones. Our previous study also detected 71 out of 85 cloacal swabs (85.5%) from healthy backyard layers (RIR breed) were positive for *E. coli* in West Bengal, India (9). Similarly, in Bangladesh, *E. coli* was isolated from 83% cloacal swabs of apparently healthy layers (24). In other countries, such as the United Kingdom, 16 *E. coli* isolates were obtained from three healthy layers and nine isolates were obtained from their feed, drinking water, and litter (41). In Australia, 59 *E. coli* isolates (85.5%) were isolated from 69 free-range egg-layer chickens (31). In India, an earlier report found 12.5% of litter samples from farmed broiler birds were positive for *E. coli* (12).

In the four studied agroclimatic zones, isolation of *E. coli* from the cloacal swabs was found to be correlated with the lower age group (1–4 wk) of birds ($P < 0.01$). The predominant bacteria in the chicken ceca was *E. coli* in broilers up to 2 wk of age, as detected by denaturing gradient gel electrophoresis (40). The highest *E. coli* isolation rate in birds (cloacal swabs) in the Terai zone, which differed significantly from other zones ($P < 0.05$), can be explained by the fact that the humid weather due to high rainfall with cyclic periods of wet and dry weather is conducive to *E. coli* growth; this was observed in the Terai zone during the sample collection period (11).

The majority of the *E. coli* isolates belonged to untypeable serogroups. Most of the serogroups of *E. coli* were observed as UT when isolated from the healthy layers in Brazil and Belgium (13,43). The serogroups isolated from healthy poultry included O2, O78, O106, rough, and UT in the United States; these were also detected in our present study (37). The serogroups O84 and O147 were also isolated from a maximum number of backyard layers in West Bengal, as reported in our previous study (9). Classical APEC serogroups such as O2 and O78 were also previously isolated from clinically healthy layer hens in Italy (3). The high frequency of detection of serogroup O8 in the Terai zone was probably due to the presence of commercial broiler farms in the vicinity that were not situated near the sample collection area of other agroclimatic zones.

We could not detect the major APEC virulence genes (*papC, tsh, iucC*) in any of the *E. coli* isolates. Similarly, *papC* was not detected in any of the 59 *E. coli* isolates from free-range healthy layers in Australia (31). On the other hand, in healthy commercial broilers *papC* (6%) and *tsh* (4%) were detected at a very low frequency in comparison to the broilers with colisepticemia in Brazil (18.5% and 39.5%, respectively) (10). However, in contrast, the presence of the *tsh* (93%) and *pap* (15%) gene with higher frequency was detected in healthy commercial broilers in Ireland (29). Our finding of the *astA*

gene in the isolates was in corroboration with other works such as Circella *et al.* (7) and Randall *et al.* (35), who also found the *astA* gene possessing APEC from the intestinal tract of healthy turkey and commercial broilers in Italy and the United Kingdom. In contrast, 20% of the APEC strains isolated from broilers with colisepticemia were also detected to possess the *astA* gene in Germany (16). It seems that *astA* is associated with an APEC virulotype from both healthy and diseased birds. In healthy birds it has pathogenic potential because it was confirmed earlier that each virulence factor alone can induce avian colibacillosis (39). It can be speculated that the low prevalence of infection among backyard chickens is, in most cases, lower than in commercial breeds and hybrids because of fewer associations of the pathogens with the required virulence genes to establish an infection (30); this could explain the absence of the major virulence genes in the present study. More related studies are required to establish the fact firmly. Further, absence of the major virulence genes (*papC, tsh, iucC*) in *E. coli* isolated from the studied backyard layers also affirmed that this kind of bird does not act as a food vehicle to spread UPEC in humans, as the same genes are shared by both of them, and the oral route may have the potential to spread the uropathogenic infection. Specifically, the plasmid (pTJ100) carrying the *tsh* and *iucC* in *E. coli* is transmissible to other *E. coli* or related bacteria and supports establishment of an extraintestinal infection (36).

We have also detected *E. coli* possessing the *astA* gene in drinking water of birds kept in the Coastal zone. In this zone, water collected from the local ponds in the vicinity was used for drinking by the birds. The ponds were otherwise used for bathing and washing after defecation by the villagers, possibly making them a source of *E. coli* that possess the *astA* gene, a common cause of watery diarrhea in humans in India (2). In other studied agroclimatic zones, ground water used for the birds to drink was free of fecal contamination.

Our study detected 18 different serogroups of *astA* gene-possessing *E. coli*. Similar types of studies regarding the virulence gene profile of *E. coli* serogroups isolated from backyard layer birds were apparently not available to compare with the present finding. However, in contrast, O2 and O78 isolated from healthy farmed broiler birds were found to possess the *tsh* gene in Italy (3). The difference in virulence gene patterns of *E. coli* isolates from the birds was probably due to differences in geographic location, breed, and the farming systems for the birds.

The RAPD analysis could not find any serogroup or agroclimatic zone–specific pattern. This is in support of the observations by Krüger *et al.* (26) and Mahanti *et al.* (28), where they found many RAPD patterns among the non-O157 *E. coli* isolates belonging to the same serotypes or locality.

In corroboration with our study, higher resistance against chloramphenicol and cotrimoxazole and zero resistance against ciprofloxacin were observed in *E. coli* isolated from free-range chickens in Nigeria (33). The majority of *E. coli* isolated from backyard layers in West Bengal in our previous study also showed sensitivity to ciprofloxacin (9). In contrast, in Australia *E. coli* isolated from free-range layers was found highly resistant against tetracycline and with zero resistance against cotrimoxazole (31). Intermediate sensitivity was observed against ampicillin, neomycin, and norfloxacin, as detected earlier in APEC isolates from Thailand (5).

The antibiotic resistance profile was statistically correlated with the agroclimatic zone of *E. coli* isolation. Probably the use of erythromycin in all of the four zones, and the use of gentamicin, tetracycline, and cotrimoxazole in the Terai and New Alluvial zones in the backyard flock owners for their own medicine or in their agricultural practices (especially tetracycline) was significantly high enough to transfer the resistance among the isolates. A similar kind of correlation between the agricultural practices, specifically spreading liquid manure in agricultural lands nearby beaches, was linked to the contamination of the beach waters by antimicrobial-resistant *E. coli* in Canada (42).

The present study did not detect ESBL-associated genes (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$) or quinolone resistance genes (*qnrA*) in any of the isolates tested. Similarly, bla_{SHV} was not observed in any of the *E. coli* isolates from studied free-range layers in Australia, although bla_{TEM} was detected at a low frequency (3/59, 5.08%), probably due to transfer from a breeder flock (31). No other study regarding the antibiotic resistance gene profile of *E. coli* isolated from backyard layers was apparently available for further comparison with the present finding. The antibiotic resistance profile is largely dependent on the usage of the antibiotics in the birds or their environment (18). The finding of no resistance genes in backyard layers in the present study could be explained by the very limited use of antibiotics of this class in these types of birds. Phenotypic resistance against chloramphenicol, cotrimoxazole, gentamicin, and tetracycline, as observed, was probably acquired from humans, from the environment due to agricultural uses, and from commercial broilers which had received similar kind of antibiotics for their treatment due to sharing of the common atmosphere (27).

Escherichia coli isolated from the backyard layers kept in different agroclimatic zones of West Bengal, India did not possess the major studied virulence genes of APEC (*papC, tsh, iucC*) required to establish infection in the birds, as well as in humans, due to similarity in the virulence factors between APEC and extraintestinal *E. coli* such as UPEC. Further, absence of ESBL and quinolone resistance genes makes the birds and their product a safe food for human consumption in comparison to commercially farmed birds.

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