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Isolation and Characterization of *E. coli* and *Comamonas kerstersii* from Chicken Litter Samples from North India

Sunil Kumar, Mukesh Yadav, Razique Anwer, Nirmala Sehrawat, Tamanna Devi, Anil Kumar Sharma

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ABSTRACT

Most of the studies involved euthanasia of chicks to investigate their intestinal microbial composition as traditional methods. But nowadays, alternative non-invasive sampling methodologies are opted to come over sacrificing the latter. Therefore, in the present study, we tried to assess the prevalence of different bacteria from chicken litter samples collected from different poultry farms and assessed antimicrobial susceptibility of the same. The recovered microorganisms are the representative of the microbiota, which are present in the ileum and caecum of the chicken, Herein, a total of 40 chicken litter and dust samples were collected in the sterile containers from four different poultry farms of Mullana territory; followed by physico-chemical and bacteriological analyses using standard methods. All the samples were cultured on nutrient broth and agar media followed by serial dilutions to determine the colony forming units (CFUs) by spread plate count (SPC) method. Molecular characterization of the bacterial isolates was performed using MALDI-TOF MS while the antibiotic susceptibility testing was performed for all the positive cultures using Kirby-Bauer disk diffusion assay. Results showed a high prevalence of *E. coli* (n=22, 64.7%) followed by *Comamonas kerstersii* (n=5, 14.7%), *Enterococcus faecium* (n=2, 5.8%). One isolate of each of *Proteus mirabilis, Bacillus pumilus, Campylobacter jejuni, Citrobacter koserii,* and *Citrobacter sedlakii* were also detected. All the isolates were susceptible to most of the anticrobial tested by disk diffusion assay. In conclusion, non-invasive sampling methods could be opted to detect the microbial community of the operational taxonomic units found in invasive samples of chicken.

Keywords Chicken Litter, *Comamonas kerstersii*, *E. coli*, *Enterococcus faecium*, MALDI-TOF MS.

Sunil Kumar [1]*
[1] Associate Professor, Department of Microbiology, Kampala International University, Western Campus, Ishaka, Uganda

Mukesh Yadav [2], Nirmala Sehrawat [4], Tamanna Devi [5], Anil Kumar Sharma [6]
[2,4] Assistant Professor, [5] Masters Student, [6] Professor and Head
[2,4,5,6] Department of Biotechnology, Maharishi Markandeshwar (Deemed to be) University, Mullana (Ambala), Haryana, India

Razique Anwer [3]
[3] Assistant Professor, Department of Pathology, College of Medicine, Imam Mohammad Ibn Saud Islamic University (IMSIU), Riyadh, SAUDI ARABIA

Email: sunilhr10h@gmail.com, sunilpgi85@gmail.com
*Corresponding author

INTRODUCTION

Chicken litter is produced in largest amount from

several types of poultry raising actions (Bolan 2010). Chicken litter is a combination of faeces, bedding material, wasted feeds, and feathers (Sanchuki 2011). This waste is prevalently used for land application as an organic fertilizer because of it's high organic content, which enhance the fertility of soil to improve the crop quality and production amount. It has nitrogen, which attribute to inherently elevated protein and amino acids levels (Enticknap *et al*. 2006). Besides its high organic content, poultry litter can harbour various types of pathogens including viruses, bacteria, parasites and fungi and various antimicrobial resistance (AMR) genes (Pizarro *et al*. 2019). A recent study indicated that direct land application of chicken litter could be harmful for animal, human, and environmental health (Kyakuwaire *et al*. 2019). Counts of pathogenic strains are also very crucial, like; *E. coli* (10^5-10^{10} CFU/gm) and Coliform bacteria (10^6-10^8 CFU/gm) are considered as exceeding the maximum permissible limits (MPLs) for land application. With the expansion of the poultry industry in all regions across the world, production of poultry litter as a waste product has also increased, further encouraging its use as manure. Therefore, it is essential to analyze its properties before it is applied to the environment like; for land application.

Indirect evidence indicated that frequent use of antibiotics linked to increase of antimicrobial resistance (AMR), the transmission of antibiotic-resistant bacteria from food animals to humans, and the environment (Gupta *et al*. 2021). Foodborne bacteria such as Salmonella, *E. coli, Staphylococcus aureus*, Enterococcus and Campylobacter spp. have also been isolated in poultry litter (Chinivasagam *et al*. 2010). Genomic analysis of novel enterococci strains has recently revealed the spread of plasmid-borne Tet (M), Tet (L) and Erm (B) genes from chicken litter to agricultural soil in South Africa (Fatoba *et al*. 2022). Avian pathogenic *Escherichia coli* (APEC) causes one of the most detrimental bacterial diseases to the United States poultry industry, colibacillosis (Fancher *et al*. 2021). Metagenomic analysis of chicken farm litter revealed the *Clostridium perfringens* amplicons by PCR analyses (PMID: 35068098). Potential environmental transmission was investigated using detection of ESBL-/AmpC-producing *E. coli* from chicken litter applied soil surface (Siller *et al*. 2021).

There are no standards guidelines available specifically for chicken litter for most of its known contaminants. Even where standards are available for allied products such as compost, there is huge variation among countries and regulating bodies for safe removal of organic wastes. As a result, this study was aimed to isolate some known and newly appeared contaminating pathogens in chicken litter, which may pose a potential risk to animal, environmental or human health. The outputs from this research could help in establishing proper program of litter management and application targeting a sustainable agriculture production.

MATERIALS AND METHODS

Sample collection

A total of 40 chicken litter and dust samples were collected from four different poultry farms randomly during January to June, 2022 in the Mullana territory of district Ambala, Haryana, North India. Approximately 20 gram of each water sample was collected in the sterile ziplock container from various locations in the territory and transferred to the microbiology laboratory for further preparation and examination. The samples were kept at room temperature until further analysis, but no longer than 2 hrs or promptly cultured. All the experiments were performed under aseptic conditions to rule out any possibility of contamination.

Enumeration and isolation of total bacteria

1 gram of each of the litter samples were mixed with 9 ml of nutrient broth (Hi-Media), which supports the growth of a range of different, followed by serial dilutions from 10^{-1} to 10^{-6} dilutions. Nutrient agar (Hi-Media) media was used to subculture the isolated individual bacterial colony by streak plate methods. Standard plate count (SPC) method was used to count number of bacteria (CFU) after incubation at 37°C for 24 hrs (Yari *et al*. 2018). Plates were incubated at 37°C for 24 h. From the culture positive plates, a loop full of culture was sub-cultured on EMB Agar plates followed by incubation at 37°C for 24 hrs. Green metallic sheen bearing bacterial colonies with mucous features were observed as *E. coli*. Further

confirmation was done with the help of a battery of biochemical tests. Every isolate was confirmed molecularly by MLADI-TOF MS.

Molecular analysis by MALDI-TOF MS

Bacterial isolates were grown on nutrient agar plates for 24 h at 37°C. Overnight grown culture was taken for further analysis by MALDI-TOF MS. We followed the procedure as already used before (Kumar *et al*. 2021).

Antibiotic resistant profiles

Kirby Bauer disk diffusion assay was follwed to determine the antibiotic resistance profile for 12 commonly used antibiotics according to the Clinical and Laboratory Standards Institute (CLSI) guidelines: amikacin, ampicillin, ciprofloxacin, cefotaxime, cefipime, colistin, ceftazidime, gentamicin, meropenem and tetracycline, norfloxacin and ofloxacin. 0.5 McFarland solution (having 1.5 x 10^8 CFU) of each of the isolate were prepared in 1N saline. A sterile cotton swab was taken and dipped into the prepared inoculums. Excess inoculums were removed from the swab by gently squeezing it against the inner side of the test tube. A sterile Mueller Hinton Agar (MHA) plate was taken for preparing a lawn culture with the help of inoculums bearing cotton swabs. The plate was allowed to dry for 5 minutes after spreading with swabs. Using sterilized forceps or needles antibiotic discs were placed on the surface of agar followed by incubation at 35°C for 24 hrs. After overnight incubation, zone diameter of antibiotic inhibition measured to interpret the resistance or susceptible types of each antimicrobial.

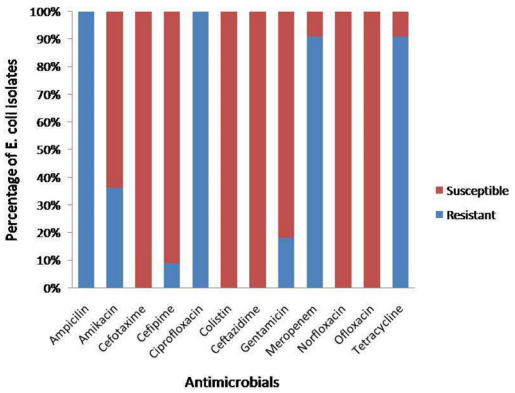

Fig. 1. Antimicrobial susceptibility profile of 22 *E. coli* isolates.

RESULTS AND DISCUSSION

A total of 34 isolates were grown after examination of 40 chicken litter samples, of which 22 (64.7%), were *E. coli*, 5 (14.7%) were *Comamonas kerstersii*, 2 (5.8%) were *Enterococcus faecium* (Table 1, Fig. 1). One isolate of each of *Proteus mirabilis, Bacillus*

Table 1. MALDI Results and antimicrobial susceptibility testing using disc diffusion assay of bacterial isolates of water.

		Antimicrobial Susceptiblity Testing											
Sl. No.	MALDI-TOF MS Results	Amp-icilin	Ami-kacin	Cefot-axime	Cefi-pime	Cipr-oflo-xacin	Col-istin	Cef-tazi-dime	Gen-tam-icin	Mer-ope-nem	Nor-flox-acin	Ofl-oxa-cin	Tetr-acyc-line
1	*Bacillus pumilus*	S	S	S	S	S	S	S	S	S	S	S	S
2	*Campylobacter jejuni*	S	S	S	S	S	S	S	S	S	S	S	S
3	*Citrobacter koserii*	S	S	S	S	S	S	S	S	S	S	S	S
4	*Citrobacter sedlakii*	S	S	S	S	S	S	S	S	S	S	S	S
5	*Comamonas kerstersii*	S	R	S	S	R	R	S	S	S	S	S	S
6	*Comamonas kerstersii*	S	S	S	S	R	R	S	S	S	S	S	S
7	*Comamonas kerstersii*	S	R	S	S	R	R	S	S	S	S	S	S
8	*Comamonas kerstersii*	S	R	S	S	R	R	S	S	S	S	S	S
9	*Comamonas kerstersii*	S	R	S	S	R	R	S	S	S	S	S	S
10	*Enterococcus faecium*	R	S	S	S	R	S	S	R	S	S	I	S
11	*Enterococcus faecium*	R	S	S	S	I	S	S	R	S	S	R	S
12	*Escherichia coli*	R	S	S	S	R	S	S	S	R	S	S	R
13	*Escherichia coli*	R	S	S	S	R	S	S	S	R	S	S	R
14	*Escherichia coli*	R	R	S	S	R	S	S	S	R	S	S	R
15	*Escherichia coli*	R	S	S	S	R	S	S	S	R	S	S	R

Table 1. Continued.

Sl. No.	MALDI-TOF MS Results	Antimicrobial Susceptiblity Testing											
		Amp-icilin	Ami-kacin	Cefot-axime	Cefi-pime	Cipr-oflo-xacin	Col-istin	Cef-tazi-dime	Gen-tam-icin	Mer-ope-nem	Nor-flox-acin	Ofl-oxa-cin	Tetr-acyc-line
16	*Escherichia coli*	R	R	S	S	R	S	S	S	R	S	S	R
17	*Escherichia coli*	R	S	S	R	R	S	S	R	R	S	S	R
18	*Escherichia coli*	R	R	S	S	R	S	S	S	R	S	S	R
19	*Escherichia coli*	R	R	S	S	R	S	S	S	R	S	S	R
20	*Escherichia coli*	R	S	S	S	R	S	S	S	S	S	S	S
21	*Escherichia coli*	R	S	S	S	R	S	S	S	R	S	S	R
22	*Escherichia coli*	R	S	S	S	R	S	S	R	R	S	S	R
23	*Escherichia coli*	R	R	S	S	R	S	S	S	R	S	S	R
24	*Escherichia coli*	R	S	S	S	R	S	S	S	R	S	S	R
25	*Escherichia coli*	R	S	S	S	R	S	S	S	R	S	S	R
26	*Escherichia coli*	R	R	S	R	R	S	S	S	R	S	S	R
27	*Escherichia coli*	R	S	S	S	R	S	S	R	R	S	S	R
28	*Escherichia coli*	R	R	S	S	R	S	S	S	R	S	S	R
29	*Escherichia coli*	R	R	S	S	R	S	S	S	R	S	S	R
30	*Escherichia coli*	R	S	S	S	R	S	S	S	S	S	S	S
31	*Escherichia coli*	R	S	S	S	R	S	S	R	R	S	S	R
32	*Escherichia coli*	R	S	S	S	R	S	S	S	R	S	S	R
33	*Escherichia coli*	R	S	S	S	R	S	S	S	R	S	S	R
34	*Proteus mirabilis*	S	R	S	S	S	R	S	S	S	S	S	S

pumilus, Campylobacter jejuni, Citrobacter koserii, and *Citrobacter sedlakii* were also detected. A total of eight types of bacterial genus were detected from 34 identified samples which have different implications in the human health (Table 2). No growth was reported in the six of the 40 processed samples. All the isolates were susceptible to all the antimicrobials (Table 1). MALDI-TOF MS correctly identified the bacteria up to species level (Table 1). CFU counts revealed that a heavy burden of bacterial load was present in the chicken litter of the territory. Although we collected the sample from a limited geographical location that too in one particular season, we were able to isolate 5 most common pathogenic bacteria (*E. coli, Comamonas kerstersii, Enterococcus faecium Proteus mirabilis* and *Campylobacter jejuni*), indicative of the alarming situation of probability of an outbreak. Out of 34 isolates, 1 isolates (*Enterococcus faecium*) belonged to coliform group in this study (Table 1). Most of the *E. coli* isolates were found resistant ampicillin, ciprofloxacin, meropenem and tetracycline however they were completely susceptible to cefotaxime, colistin, ceftazidime, norfloxacin and ofloxacin (Fig. 1, Table 1). All the *C. kerstersii* isolates were resistant to ciprofloxacin and colistin (Table 1).

In the last five years, only two studies were available on PubMed on this line. A study from the same state Haryana, North India has isolated 62 *E. coli* isolates (54%) from a total of 114 samples of broiler chicken and environmental samples (Grakh *et al*. 2022). They identified them as avian pathogenic *Escherichia coli* (APEC), which is responsible for colibacillosis in poultry. 91% of the APEC isolates in that study were multidrug resistant (MDR). Another study from Kerala, South India has isolated 31 *E. coli* (64.5%) from 48 chicken litter samples recently. All the isolates in this study were resistant to ampicillin, meropenem amoxicillin, and tetracycline. More than 40 % of the *E. coli* isolates were resistant to amikacin, cefotaxime and ofloxacin (Sebastian *et al*. 2021).

Comamonas kerstersii is a Gram-negative bacillus, which is ubiquitous in the environment and rarely causes human disease. There is very scarce literature available on *C. kerstersii* due to non-reliable identification tests. First case of human infection due to *C. kerstersii* was reported in 2013. Till date, only 18 articles are reported all over the globe (PubMed search) on human diseases caused by *C. kerstersii*. First complete genome of *C. kerstersii* 8943 was published recently in 2018 (Jiang *et al*. 2018). In

Table 2. Type of bacterial isolates in different specimens and their characteristics.

Sl. No.	Bacteria	Total no.	Properties
1	*Escherichia coli*	22	Commonly found in the lower intestine of warm-blooded organisms but some strains can cause diarrhea and vomiting
2	*Comamonas kerstersii*	5	Associated with severe diseases such as abdominal infection and bacteremia.
3	*Enterococcus faecium*	2	*E. faecium* has been seen as commensals of the gastrointestinal tract but recently evolved as a worldwide nosocomial pathogen.
4	*Bacillus pumilus*	1	It resides in soils and some colonize in the root area of some plants.
5	*Campylobacter jejuni*	1	It is one of the most common causes of food poisoning in Europe and in the US.
6	*Citrobacter koseri*	1	Frequently found in water, soil, food, and the intestines of ani mals and humans. Low virulent *C. koseri* can cause life threatening infections in the immunocompromised patients especially in the neonates.
7	*Citrobacter sedlakii*	1	It has been described as causing human disease, originally isolated from human stool and wounds but is generally found as a non-pathogenic organism in human stools.
8	*Proteus mirabilis*	1	*P. mirabilis* widely distributed in soil and water and causes 90% of all proteus infections in humans.

recent years, application of MALDI-TOF MS has enabled quick and reliable laboratory identification of *C. kerstersii*. Hence, many cases of bacteremia have been reported recently due to this bacterium from China, Canada and Uruguay (Liu *et al*. 2020, Palacio *et al*. 2020, Rong *et al*. 2022). In India, no previous report from any environmental or clinical samples is available on *C. kerstersii*. Present study showed the isolation of 5 strains of *C. kerstersii* for the first time in India and interestingly, all were resistant to ciprofloxacin and colistin.

A recently published study showed the detection of *Proteus mirabilis* from poultry house dust, excreta and litter in the Victoria region of Australia (Bindari *et al*. 2021). Our study also revealed an isolate of *Proteus mirabilis* from chicken litter samples. Standards local and global, easy-to interpret and measurable methods of analysis and safely application of chicken litter as an organic fertilizer must be implemented for land application and general discharge into the environment. This study suggested the frequent surveillance of chicken litter before and after the application in the soil to fix any spread of harmful bugs into the environment.

CONCLUSION

The current study displayed the presence of many antimicrobial resistant bacteria in the chicken litter, which suggested the probable dissemination of the same in to environment and humans. This is a potential threat for human and environmental health. Relatively, a very high percentage of *E. coli* isolates were recovered from poultry farms samples, which were resistant to many clinically relevant antibiotics, like; meropenems, ampicillin and tetracycline. There were 5 isolates of *C. kerstersii*, which has been seen in a number of bacteremia cases in the hospital settings. All the *C. kerstersii* isolates were resistant to ciprofloxacin and colistin. This is the first study from India where, *C. kerstersii* has been traced for the first time in the chicken litter samples. Certainly, further studies should be performed in the Mullana territory for more data by performing molecular techniques, like; MLST, PFGE or Whole Genome Sequencing (WGS), to study their phylogeny and genetic relatedness among environmental and clinical isolates.

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