British Microbiology Research Journal
14(5): 1-9, 2016, Article no.BMRJ.25883
ISSN: 2231-0886, NLM ID: 101608140

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Multidrug Resistant *Providencia stuartii* in Chicken Droppings: Public Health Implications for Poultry Workers and Associated Communities in nearby Dhaka Metropolis, Bangladesh

Arifatun Nahar[1], Mahfuza Marzan[1], Mashuk Siddiquee[1], Shamsun Nahar[1], Kazi Selim Anwar[2] and Salequl Islam[1*]

[1]Department of Microbiology, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh.
[2]Microbiology Unit, Faculty of Medicine, AIMST University, Malaysia.

Authors' contributions

This work was carried out in collaboration between all authors. Authors SI and AN designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Authors MM, MS, SN and KSA managed the data acquisition and analyses of the study and literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2016/25883
Editor(s):
(1) Ana Cláudia Coelho, Department of Veterinary Sciences, University of Trás-os-Montes and Alto Douro, Portugal.
Reviewers:
(1) Otolorin Gbeminiyi Richard, University Zaria, Nigeria.
(2) Shalini Malhotra, Delhi University, India.
Complete Peer review History: http://sciencedomain.org/review-history/14507

Original Research Article

Received 24th March 2016
Accepted 23rd April 2016
Published 7th May 2016

ABSTRACT

Introduction: Poultry farms (PFs) have appeared successful and wide spread business-industry in Bangladesh, which often remains contaminated with various hazardous microorganisms when standard hygiene practices are compromised. We sought to investigate a zoonotic human pathogen, *Providencia stuartii* and their antibiotic resistance pattern in chicken droppings collected from local poultry farms in Savar area, Dhaka, Bangladesh.
Materials and Methods: We conducted a cross-sectional study to find the prevalence of antibiotic resistance in bacteria from chicken droppings in linked to antibiotic-uses and abuses in PFs. Random chicken droppings were collected from broiler type chickens, layer-chickens, and pre-starter broiler chickens to make samples representative. Following standard bacteriological culture,

**Corresponding author: E-mail: salequl@gmail.com, salequl@juniv.edu;*

semisolid chicken-droppings were diluted aseptically, enriched in buffered peptone water, and then streaked onto a xylose-lysine-deoxycholate agar plate. Selected *P. stuartii* colonies were identified biochemically using API 20E (*BioMe´rieux*) and antimicrobial susceptibility testing was performed following Kirby-Bauer (disk-diffusion) method.

Results: We reported the isolation of *P. stuartii* for the first time in Bangladesh in chicken droppings collected from randomly selected local PFs. Red colored colonies without black centre on XLD medium were considered as presumptive *Providencia stuartii* that were subsequently confirmed by API 20E system. Six chicken-droppings revealed the presence of *P. stuartii* from a total 70 samples tested, showed a prevalence of 8.6% with overall farm prevalence is 71.4%. We took 11 isolates from the six positive samples to examine their antibiotic resistance and found 82% of them were resistant to nalidixic, 73% to ampicillin, and 54.5% to trimethoprim- sulfamethoxazole. Relatively ciprofloxacin and gentamicin appeared more functional, where only 27.3% and 18.2% strains showed resistant, respectively. Over 54% of the isolates appeared resistant to >3 antibiotics and 36.4% with two different antibiotics. None of the isolates remained susceptible to all the 6 antibiotics tested.

Conclusion: Detected MDR *P. stuartii* in chicken-droppings from local poultry farms may contribute their transmission to surrounding communities and may implicate serious biosecurity concern in environmental and food-safety issues in resource constraint countries, like, Bangladesh.

Keywords: Poultry; Providencia stuartii; multidrug-resistance; Bangladesh.

ABBREVIATIONS

API : Analytical Profile Index
CLSI : Clinical and Laboratory Standards Institute
MDR : Multidrug Resistant
MHA : Mueller-Hinton Agar
PBS : Phosphate Buffered Saline
PFs : Poultry Farms
UTI : Urinary Tract Infection
WHO : World Health Organization
XLD : Xylose Lysine Deoxycholate

1. INTRODUCTION

Poultry farms (PFs) have commenced as one of the most wide-spread industries in Bangladesh because of their low-investment requirements [1]. These PFs generate high employment opportunities in poultry value chain [2], thereby, are contributing to national economy notably. Consequently, broiler meats and layer farm eggs fulfill the maximum demand of animal protein for the entire population of Bangladesh [2,3]. Apart from these, most of the PFs in Bangladeshi are run by unskilled staff and non-professional management having lack of proper knowledge on concerned biosecurity, environmental hygiene practices and food safety regulations [4]. These farms neither possess a robust surveillance system nor a stringent regulatory body. Even these PFs don't have a well-documented data archiving mechanism to record pathogenic microorganisms, systematically [5]. Bangladeshi poultry sectors usually do misuse and/or over-use of several wide-spectrum antibiotics and thereby, have generated selection pressure towards antimicrobial resistance [6]. However, this important aspect is often ignored or overlooked in Bangladesh [7] and some other countries [8-10]. The World Health Organization (WHO) is much concerned on the spreading of antibiotic resistance in the South-East Asian countries, where medicine uses are not very strict [11].

Bangladesh is a small and resource-constraint country that suffers from serious environmental pollution. The country lacks decent sewerage management and water sanitation systems, particularly in sub-urban and village areas. Practices exist of spreading chicken droppings into nearby environments from PFs that may disseminate potential zoonotic pathogens into the surrounding communities quite easily. High dense population may also augment transmitting the infections person-to-person and aggravate in threatening public health. Hazardous microorganisms that are reported from Bangladeshi poultry contamination include *Enterobacter spp.*, *Escherichia coli* and non-*Salmonella enterica*, *Proteus mirabilis* and other species of *Enterobacteriaceae* [12-14]. We sought to investigate the zoonotic pathogen, *Providencia stuartii* in chicken droppings collected from local poultry farms under this study. The genus Providencia are opportunistic pathogens that include six species, namely, *P. stuartii, P. rettgeri, P. alcalifaciens,*

P. rustigianii, P. heimbachae, and P. vermicola [15]. They are Gram negative, facultative anaerobe and motile bacteria, reportedly have multiple animal reservoirs. Providencia *spp*. were reported to cause a large outbreak of foodborne illnesses [16], pericarditis [17], meningitis [18], endocarditis [19] and diarrhea [20]. *P. stuartii* remains the second most common cause of catheter-associated urinary tract infection (Ca-UTI) and, was reported to co-colonize with *P. mirabilis* [21,22]. To our knowledge, there is no baseline data available yet on the contamination rate and antimicrobial resistance pattern of *P. stuartii* in Bangladeshi PFs/chicken droppings. Therefore, this study was a footstep initiative to fill up the knowledge gap. The isolation of *P. stuartii* from poultry source will authenticate formulating awareness program for preventing zoonotic infections to vulnerable workers and associated communities in Bangladesh.

2. MATERIALS AND METHODS

2.1 Description of Study Area and Collection of Samples

The fast growing PFs have flourished in both urban and rural areas of Bangladesh and abundantly around capital city, Dhaka. More than half of the PFs in the country are situated at the periphery of Dhaka district. We conducted this study in one of its sub-district 'Savar'- located nearly 30 km north-west of Dhaka city (Fig. 1). Seventy chicken droppings were sampled (10 each of 7 PFs surveyed) from structurally-different semi-urban areas and a unique identification code was assigned for each sample based on area-specific individual PFs (Table 1). We conducted a cross-sectional study to find the prevalence of antibiotic resistance in bacteria from chicken droppings in linked to antibiotic-uses and abuses in PFs. Representative chicken droppings were collected from broiler type chickens, layer-chickens, and pre-starter broiler chickens from different racks randomly. The sampling PFs were selected from two different communities of Savar area to cover the interpretive population.

2.2 Preparation of Samples

Following all safety precautions and aseptic techniques, chicken droppings were collected to avoid probable cross contaminations. Samples were stored immediately in pre-labeled clean plastic containers in insulated ice-boxes and transported to the bacteriology lab of the Department of Microbiology, Jahangirnagar University, Savar, where all microbiological tests were carried out. One loopful of semisolid chicken dropping was diluted aseptically within 200 µl sterile phosphate buffered saline (PBS) in micro-centrifuge tubes and vortexed to mix homogenously, and then, inoculated on pre-selected culture media. The remaining sample-portions were stored overnight safely at 4°C to repeat the microbiological analyses.

2.3 Culture and Identification Techniques

For efficient bacteriological culture, we adopted modified techniques of traditional *Salmonella* isolation protocol to for isolating *Providencia stuartii.* Briefly, the diluted chick-droppings were enriched in buffered peptone water (HIMEDIA, India), and one loopful of broth was streaked onto Xylose lysine deoxycholate (XLD) agar plates (MERCK, India). After an overnight incubation (24 h at 37°C), three to four *Salmonella*-like typical colonies (reddish butt with/without a black dot in centre) were picked-up with flame-sterilized needle and stab-streaked into nutrient agar slants to examine specific phenotype characteristic of swarming growth by Proteus species. The purified colonies were identified further based on detailed biochemical properties for the colonies of *Providencia stuartii* using a rapid biochemical-test kit (API 20E*, BioMe´rieux*, Durham, NC) consisting of a set of chromogenic panel, carbohydrate batteries and enzymatic substrates [23].

2.4 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) for *Providencia stuartii* isolates was performed following the standard Kirby-Bauer disk diffusion method [24] and zones of inhibition were interpreted following CLSI (Clinical & Laboratory Standards Institute-2010) recommendations. Six different antimicrobials, which are commonly used by Bangladeshi poultry farmers [7], were tested against *P. stuartii* in order to characterize their resistance potential. Antimicrobials belonged to five different genera were tested (beta-lactam, quinolone, tetracycline, aminoglycosides, trimethoprim- sulfamethoxa-zole) in this study.

P. stuartii isolates grown on XLD were first transferred into nutrient agar and incubated overnight at 37°C to get pure culture. Then, one loopful of *inocula* was added onto 9 mL Mueller-

Hinton (MH) broth and incubated aerobically at 37°C for 5–6 hours so as to get a turbidity equivalent to 0.5 McFarland standard. Then, these inocula were lawned homogenously with a sterile cotton swabs on the MHA plates (Oxoid, UK). After air drying under a safety hood,

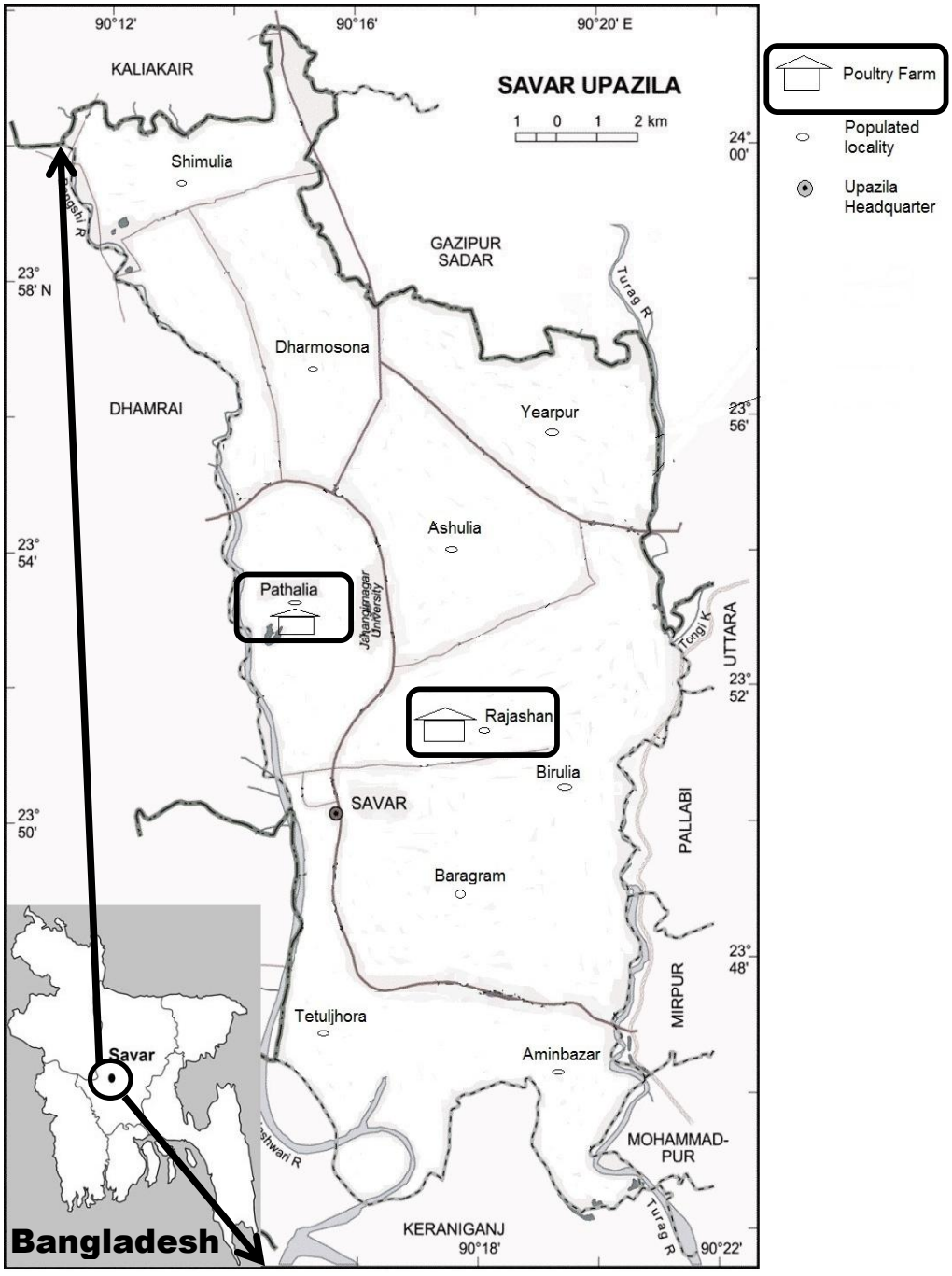

Fig. 1. Location of sampling sites in the map of Bangladesh

A total of 70 chicken dropping samples were collected from seven poultry farms located at Savar area, which is located about 30 kilometer north-west of the capital city of Bangladesh, Dhaka. Savar is semi-urban area that have been established with different scale poultry farms and other industries. Out of the seven sampling farms, three were cultivating broiler type chickens, three with layer-chickens and one with pre-starter broiler chickens

antibiotic discs (amoxicillin, 10 µg, Ciprofloxacin, 5 µg, Gentamicin, 10 µg, Nalidixic acid, 30 µg, Tetracycline, 30 µg and trimethoprim-sulfamethoxazole, 25 µg) were placed on those evenly-lawned MHA-plates, aseptically. Then, the plates were kept at 4°C for 30–60 minutes for adequate diffusion and then incubated overnight keeping upright position at 37°C aerobically. The diameter of the zone of inhibition of each disk was read to interpret as resistant, moderately sensitive or sensitive (susceptible) employing standard reference data provided by CLSI.

3. RESULTS

3.1 Isolation, Identification and Confirmation of *Providencia stuartii*

Red colored colonies without black centre on XLD medium were considered as presumptive *Providencia stuartii* resembling *Salmonella spp*. (Supplementary Fig. 1a), whereas *E. coli* showed yellowish colonies without black-centre as the standard negative control (Supplementary Fig. 1b).

Of the 81 Salmonella-typical colonies (on XLD) examined, 11 were identified as *Providencia stuartii* based more on their detail biochemical properties: slant alkaline (red), butt acidic (yellow), H_2S negative, gas negative; indole positive; methyl red positive; VP negative; citrate test positive; urease positive/negative; oxidase negative; catalase positive. Subsequently, they were further confirmed as *Providencia stuartii* employing API 20E system (Supplementary Fig. 1c). All the isolates were Gram negative and short rod shaped under light microscope.

3.2 Prevalence of *P. stuartii*

Culture findings revealed the presence of *P. stuartii* among the chicken-droppings in five of seven PFs, thus yielding an overall farm prevalence for *P. stuartii* as 71.43% (Table 1). All the samples designated CK011-CK020 and CK051-CK060 collected from PF2 and PF6 respectively showed abundant growth for typical faecal microorganisms in both aerobic and anaerobic conditions. However, no growth of *P. stuartii* were found in PF2 cultivating layer-type chicken (located in '*S. Rajason'* area), and PF6 cultivated pre-starter broiler-type chicken (located in '*Pandhoya'*). Samples from remaining farms revealing the growth of *P. stuartii* demonstrated an intra-sample prevalence of 10% in PF1, PF4, PF5 and PF7 to 20% in PF3 (Table 1). Thus, the overall prevalence of *P. stuartii* among 70 chicken-droppings examined was about 9%, (6/70), when 11 of the isolates from 6 positive-samples were examined for subsequent AST.

3.3 Resistance Patterns of *P. stuartii*

The antimicrobial drug sensitivity against 11 isolates of *P. stuartii* showed the highest resistance (82%, 9/11) to nalidixic acid, followed by amoxicillin (73%, 8/11) (Table 2). High levels of resistance were also exhibited against trimethoprim- sulfamethoxazole (54.5%, 6/11). In contrast, *P. stuartii* isolates showed relatively low resistance (27.3%, 3/11) to ciprofloxacin and gentamicin (18.2%, 2/11), (Table 2). The sensitivity of representative *Providencia stuartii* isolates were demonstrated by the clear zones of growth inhibition around the discs by the Kirby-Bauer technique (Supplementary Figs. 1d, 1e).

Combining these findings, 54.5% (6/11) *P. stuartii* were observed to be MDR being resistant to >3 antibiotics from different generic-groups (Fig. 2). MDR with two different antibiotics were observed among 36.4% (4/11) isolates contrary to only 9% (1/11) being resistant to a single antibiotic. However, none of the *P. stuartii* isolates remained susceptible to all the 6 antibiotics tested.

Table 1. Prevalence of *Providencia stuartii* in chicken droppings

Poultry farm [a]	Sample(s)	No. (%) of positive samples
F1	CK001-010	1 (10)
F2	CK011-020	0 (0)
F3	CK021-030	2 (20)
F4	CK031-040	1 (10)
F5	CK041-050	1 (10)
F6	CK051-060	0 (0)
F7	CK061-070	1 (10)
Total	CK001-070	6 (8.6)

[a,] *Ten samples of chicken droppings were collected from each of the seven different farms located in sub-urban area of Savar, Bangladesh*

Table 2. Antimicrobial sensitivities [a] of *Providencia stuartii* isolated from chicken droppings

Antimicrobial agent	Sensitivity category, number (%) of isolates, n=11		
	Sensitive [b]	Moderate sensitive	Resistant
Ampicillin	2 (18.2)	1 (9.1)	8 (72.7)
Ciprofloxacin	8 (72.7)	0 (0)	3 (27.3)
Gentamicin	8 (72.7)	1 (9.2)	2 (18.2)
Nalidixic acid	2 (18.2)	0 (0)	9 (81.8)
Tetracycline	2 (18.2)	4 (36.4)	5 (45.4)
Trimethoprim + Sulfamethaxole	3 (27.3)	2 (18.2)	6 (54.5)

[a] *Antimicrobial susceptibility testing for Providencia stuartii isolates was done following Kirby-Bauer disk diffusion method*

[b] *Results were interpreted according to instructions from the Clinical and Laboratory Standards Institute (CLSI, 2010)*

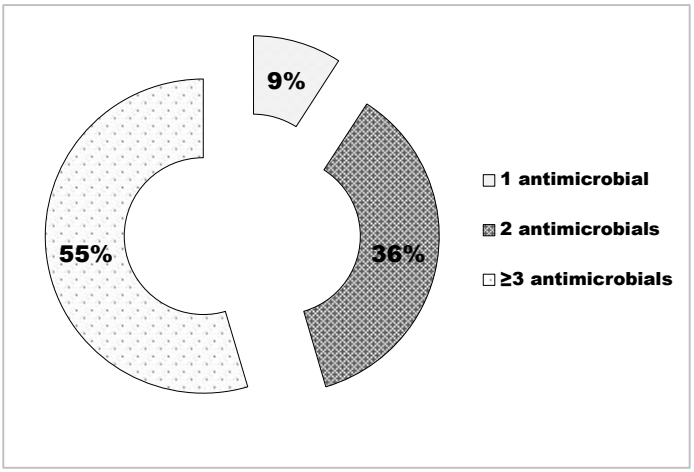

Fig. 2. Prevalence of multidrug-resistant *Providencia stuartii* isolated from chicken droppings of Bangladeshi poultry farms

MDR was characterized when single Providencia stuartii isolate had shown resistance to three or more antimicrobials of different groups. Accordingly, about 55% of Providencia stuartii were MDR; about 36% isolates were resistant simultaneously to two different antimicrobials and only 9% were found resistant to single antimicrobial

4. DISCUSSION

The growth of poultry production by setting up chicken farms has increased extensively over the last 15 years in developing countries including Bangladesh [5]. PFs presumly contribute in the dissemination of various zoonotic pathogens, thus, spreading in working areas and surrounding communities leading to potential public health hazards [8,9]. Parallel to the rapid expansion of the business, sufficint biosecurity measures are not being practiced in these Bangladeshi PFs despite existing guideline on the prevention and control of transmission of associated pathogens [25,26]. A few studies have been carried out to identify zoonotic pathogens in Bangladeshi PFs [13,14,27]. There was a clear knowledge gap about many potential zoonotic pathogens that could be transmitted via poultry industries [28,29]. The promising poultry industry in Bangladesh may emerge as potential epicenters of zoonotic epidemics in absence of proper attentions and care. The reporting of *Providencia stuartii* from the poultry samples will be a flagship findings in this arena for the country.

Over 55% of the *P. stuartii* isolates became MDR against six most commonly used antibiotics we tested in this study. Most of these antibiotics are often used by the PF-workers/farmers to treat chickens in Bangladesh, including erythromycin and streptomycin [7] and often in a combination of two or more antibiotics. These aforementioned facts remain one of the most prudent contributing factors in acquiescing emergence of

antimicrobial resistance in chicken-population in adjunct to certain selective pressure owing to indiscriminant use of antibiotics in these PFs [30]. Notably, antibiotics are easily available in Bangladesh beyond prescriptions that dispense without proper dosage, regimen directions of usage to treat human patients or to protect diseased poultry-flocks [11,31]. These misuses were reported to promote emergence of MDR strains in many countries [32]. Earlier studies reported *P. stuartii* to be resistant to penicillins, aminoglycosides, tetracyclines, sulphamethoxazole, polymyxin B, colistin, nitrofurantoin, older-cephalosporins, fosfomycin and chloramphenicol [33,34]. Our antibiotic resistance outcome is congruent to the earlier research that concedes *P stuartii* as the most resistant species in *Providencia* genus [35]. Their multidrug-resistant trait is plasmid mediated [35], however, further study will be required to affirm it for our isolates.

Sub-standard sanitation practice in a improvised and densely populated country like Bangladesh may aggravate the antibiotic resistance lineage easily to population level. So, MDR *P. stuartii* in chicken droppings may aggravate spreading both by person-to-person transmission [17-19] and via human to animal interface [36]. They may impose a threat of cross-resistance to other pathogens [37], by changing in ways that lead to the reduced effectiveness of antibiotics to cure or prevent infections. Consequently, attributes of MDR-resistance may also emerge in other dominant common flora under the selective pressure of antibiotics and lead a serious clinical implications. Transmitting the PF-origin MDR-*Providencia* spp. into surrounding environment and adjacent communities could make a huge public health challenge for Bangladesh in near future as happened in other communities [35,38]. Therefore, Government health authority should administer stringent biosecurity measures and formulate national guidelines for controlling MDR and abuses of antibiotics in animal husbandry.

This study has several inherent limitations. All of the chicken dropping samples in the study were collected from Savar area (Dhaka). Samples from other areas of the country were not included in this study. The results obtained from this analysis might appear some variation to the samples in other regions of the country. The small sample size was a major constraint to convey meaningful outcomes by statistical analyses. Therefore, a prospective design of longitudinal study involving larger sample from diverse geographic areas may provide more detail insight of *P. stuartii* as zoonotic pathogens. The identification of *P. stuartii* was done based on detail biochemical followed by API 20E confirmation. However, parallel molecular detection could provide extra strength in our findings. Moreover, the establishment of a clonal relationship between PF-originated and a clinical sample remains important to translate our preliminary findings into clinical applications more effectively.

5. CONCLUSION

This study has provided baseline prevalence data on the presence of *P. stuartii* and their antimicrobial resistance pattern in the chicken droppings in poultry farms in a region of Bangladesh. The report will contribute initial findings to make awareness on the emerging zoonotic infections by *P. stuartii* in PFs in Bangladesh. More in-depth study covering other regions of the country can authenticate our preliminary findings and can be helpful in developing biosecurity concern in environmental and food-safety issues nationwide and in abroad.

ETHICS STATEMENT

Consents were obtained from respective farm owners before the collection of respective chicken droppings.

ACKNOWLEDGEMENT

This work was supported in part by the University Grant Commission (UGC), Bangladesh.

We are thankful to Higher Education Quality Enhancement Project (HEQEP), CP No. 3413, awarded to the Department of Microbiology, Jahangirnagar University, for supporting article processing fee of this publication".

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/14507