

Original Research Article

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Isolation, Identification and Characterization of Enteric Bacteria from Post Weaning Diarrheic Pigs and their Resistance to Multiple Antibiotics

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ABSTRACT

In the present study, 25 diarrheic faecal samples/ rectal swabs collected from post weaning pigs were examined by cultural method for the presence of Enterobacteriaceae members which resulted in isolation of 18 lactose fermenter and two non-lactose fermenter bacterial colonies. The recovered colonies were subjected to VITEK[®]2 Compact System for the identification of bacteria. Out of 18 lactose fermenter bacterial colonies, 15 were identified as *Escherichia coli*, two as *Enterobacter asburiae*, and one as *Klebsiella pneumoniae*. Both the lactose non-fermenter bacterial colonies were identified as *Pseudomonas aeruginosa*. In-vitro antibiotic sensitivity pattern of *E. coli* isolates showed 100% sensitivity to chloramphenicol, gentamicin and ampicillin/sulbactam whereas 60% isolates were found to be resistant to co-trimoxazole and 53% isolates to amoxicillin clavulanate and cefotaxime. All *E. coli* isolates showed resistance to more than four antibiotics, four isolates showed resistance to six antibiotics and two isolates showed resistance to more than seven antibiotics. Isolates of *K. pneumoniae*, *Enterobacter asburiae* and *P. aeruginosa* found to be sensitive to most of the antibiotics tested; whereas these isolates showed resistance to amoxicillin/clavulanate and co-trimoxazole. This study helps in assessment of bacterial resistance of enteric bacteria recovered from diarrheic pigs.

Keywords

Antibiotic resistance, *E. coli*, Post weaning diarrhea, Swine production, VITEK[®]2

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Introduction

Diarrhea in pigs is one of the major problems worldwide leading to huge economic losses to swine industry. Neonatal and post-weaning diarrhea (PWD) is responsible for high morbidity and mortality where mortality may reach up to 20-30% during acute outbreaks of PWD (Amezcuca *et al.*, 2002). Many viral, bacterial, and parasitic agents are involved in the cause of neonatal diarrhea such as

transmissible gastroenteritis (TGE) virus, swine enteric coronavirus disease (SECD) including porcine epidemic disease virus (PEDV) and swine delta corona virus (SDCV), rotavirus, *E. coli*, *Salmonella*, *Clostridium perfringens* type C, *Strongyloides ransomi* and *Coccidia*. PWD, however, is a multifactorial as it involves interaction between sow, piglet, environment, ETEC bacteria and managerial practices (Hong *et al.*, 2006). Though exact cause has not yet

been identified for PWD in pigs (Jensen *et al.*, 2006), *E. coli* is considered as main bacterial cause for PWD which can cause infection alone or in combination with rotavirus. Neonatal and post weaning diarrhea or enteric colibacillosis alone may result in heavy economic losses due to decreased weight gain, cost of treatment, vaccination, use of feed supplements and mortality (Fairbrother *et al.*, 2012). In India, the most common cause of morbidity and mortality in piglets is diarrhea (Sinha *et al.*, 2018) and *E. coli* is primarily associated with these diarrheic pigs as reported previously (Begum *et al.*, 2014; Sikdar, 1991; Begum *et al.*, 2014; Regon *et al.*, 2014; Borah, 1994; Shome *et al.*, 2005). To control PWD, antimicrobials are widely used in piggeries for prophylactic and therapeutic purposes. The most commonly used antimicrobials to treat affected piglets are amoxicillin/clavulanate, trimethoprim/sulphonamide, enrofloxacin, colistin, apramycin, ceftiofur, neomycin and gentamicin (Fairbrother *et al.*, 2012). Frequent and long-term use of a particular antibiotic in a specific region may results in the development of resistance in the bacteria to the particular antibiotic (Moon *et al.*, 2007). The multiple antibiotic resistance strains of *E. coli* emerged as formidable threat to piggy industry (Mathew *et al.*, 1999) and may spill over to other livestock as well as human population (Osterberg *et al.*, 2016; Valiakos *et al.*, 2016). Therefore, there is a constant need to update the antibiotic sensitivity profile of *E. coli* isolated from piggeries, particularly from diarrheic pigs, to help in selection of effective antibiotics for therapeutic purposes, to reduce treatment cost, and to combat antibiotic resistance.

In the present study, Enterobacteriaceae members were isolated from the post weaned diarrheic pigs, characterized using VITEK®2 system and subjected to antibiotic susceptibility testing to assess the sensitivity/resistance of isolates.

Materials and Methods

Collection of samples and processing

A total of 25 fecal samples/rectal swabs were collected from piglets from pig farms from Uttar Pradesh and Haryana, with clinical signs of diarrhea, dehydration and decreased weight gain. The samples were collected with sterile swabs under aseptic conditions and transported to the laboratory immediately under cold conditions. These samples were then diluted using 0.9% normal saline solution and processed for the isolation of bacteria.

Isolation and characterization of bacteria

Normal saline diluted fecal samples were streaked on MacConkey Lactose Agar (MLA) for the isolation of members of Enterobacteriaceae family. The plates were then incubated at 37°C for 24 hours under aerobic conditions.

The isolated colonies were subjected to Gram's staining. The lactose fermenter and lactose non-fermenter colonies were further subcultured to obtain pure colonies. The purified lactose fermenter colonies were then streaked on eosin methylene blue (EMB) agar and lactose non-fermenter colonies on brilliant green agar (BGA) for further characterization.

Microbial identification using VITEK®2 Compact System

Gram negative (GN) reagent cards having 64 wells each containing an individual test substrate were used. A sufficient number of colonies of pure culture of microorganisms were suspended in 3.0 ml of sterile saline (aqueous 0.50% NaCl, pH 4.5 to 7.0) in a 12×75 mm (polystyrene test tube). The turbidity was adjusted accordingly (for Gram negative bacteria, McFarland turbidity range should be 0.50-0.63) and measured using a

turbidity meter, DensiCheck™ and the cassettes with tubes and reagent cards were loaded in VITEK®2 Compact system and the results were interpreted as per instructions of manufacturer (BioMérieux, Inc. USA).

In- vitro antibiotic sensitivity

In vitro single disc diffusion technique (Bauer *et al.*, 1966) was employed using antibiotic discs viz., amoxicillin/clavulanate (20/10 mcg), ampicillin/sulbactam (10/10 mcg), cefoperazone (75 mcg), cefotaxime (30 mcg), chloramphenicol (30 mcg), ciprofloxacin (5 mcg), cotrimoxazole (25 mcg), gentamicin (10 mcg), kanamycin (30 mcg), levofloxacin (5 mcg), moxifloxacin (5 mcg) and tetracycline (30 mcg) for assessment of antibiotic susceptibility testing. The plates were incubated at 37°C for 24 hours under aerobic conditions. The results were then interpreted on the basis of inhibition zone as per the manufacturer's recommendations (Hi-Media, India).

Results and Discussion

Out of 25 samples processed, 18 recovered isolates exhibited lactose fermenter pink colonies and two recovered isolates exhibited non-fermenter pale colonies on MLA agar (Fig. 1A and B). Of 18 lactose fermenters, three had mucoid appearance and rest had flat colonies with entire margins. All 18 isolates were found to be Gram negative rods under oil immersion of microscope upon Gram's staining (Fig. 1C and D; Table 1). These 18 lactose fermenter isolates when streaked on EMB agar showed variable results. Twelve isolates showed green metallic sheen (Fig. 1E), five isolates showed flat, dark colored colonies and one isolate showed dark colored, mucoid colonies with no metallic sheen. The two non-lactose fermenter colonies on BGA showed pinkish, round colonies (Fig. 1F).

The lactose fermenter isolates with and without green metallic sheen on EMB agar were confirmed as *E. coli* by VITEK®2 Compact System. Of confirmed 15 bacterial isolates to be of *E. coli*, only 12 isolates showed green metallic sheen on EMB agar and three did not. The probable reason behind lack of green metallic sheen may be due to transition of *E. coli* from smooth to rough variant where bacteria lose some of its morphology and other characters. The results of the study indicated that frequency of isolation of *E. coli* from fecal samples was 60%. Primarily work on porcine *E. coli* had been carried out in north eastern state of India where pigs are raised in larger number. Isolation frequency varied in north eastern state of India ranging from 15% (Begum *et al.*, 2014), 51.95% (Sikdar, 1991), 59.3% (Begum *et al.*, 2014), 60% (Regon *et al.*, 2014), 86.11% (Borah, 1994), and 90% (Shome *et al.*, 2005). Similarly, pigs are raised extensively in Asian countries such as China, Taiwan, Japan and Vietnam where the outbreaks of PWD are quite common (Lee, 2015). The present study is quite close to results of Japan where *E. coli* was isolated from 58.1% of weaned piglets (Katsuda *et al.*, 2006). *E. coli* from weaned piglets were isolated at a lower frequency at 17.6% and 22.4% respectively in Germany and Canada (Morin *et al.*, 1983; Wieler *et al.*, 2001). The variation in *E. coli* isolation rate could be explained by geographical location, type of antibiotic usages, age of the animals, concurrent infections and no. of samples tested. Two of the bacterial isolates were confirmed as *Enterobacter asburiae* and one isolate as *Klebsiella pneumoniae*. There are no reports of *Enterobacter asburiae* causing diarrhetic symptoms in pigs. *Klebsiella pneumoniae* subspp. *pneumoniae* is a commensal as well as the cause of septicemia in pigs (Bidewell *et al.*, 2018). Though *Klebsiella pneumoniae* have not been directly reported from diarrhetic pigs but have been able to experimentally induce diarrhea in pigs

(Wilcock, 1978). The two isolates which were non-lactose fermenter on MLA and gave pinkish red colonies on BGA were confirmed as *Pseudomonas aeruginosa* using VITEK®2 System. On Mueller Hinton agar plates, the organism also produced green pigment. There are no published reports of *P. aeruginosa* being involved in porcine diarrhea. So, mere isolation of any bacteria could not be attributed to diarrhea. Even though normal and apparently healthy pigs also yield *E. coli* in their feces but the presence of *E. coli* in cases of diarrheic cases could not be understated as it is a part of multifactorial etiology of PWD. However the pathogenic nature of the isolates needs to be established using molecular characterization of various virulence gens such as toxin, bundle forming pilli etc. Overall, in the present study, 20 (80%) bacterial isolates were recovered from 25

diarrheic pigs, with *E. coli* in majority of cases (60%).

In the present study, out of 12 antibiotics assessed, 15 *E. coli* isolates showed multiple drug resistance to nine antibiotics (Table 2 and Fig. 2). There was more than 50% resistance to five antibiotics viz. co-trimoxazole, amoxicillin clavulanate, moxifloxacin, cefoperazone and cefotaxime with maximum resistance of 60% was observed for co-trimoxazole followed by amoxicillin clavulanate and cefotaxime (53.3%), moxifloxacin and cefoperazone (40%), tetracycline (33.3%), ciprofloxacin (23.3%), levofloxacin (20%) and kanamycin (13.3%). The antibiotic susceptibility of two isolates of *P. aeruginosa*, 2 isolates of *E. asburiae* and the only isolate of *K. pneumoniae subsp pneumoniae* was also determined.

Table.1 Prevalence of *E. coli* and other bacteria isolated from 25 diarrheic faecal samples of pigs

Bacteria	Number of bacterial isolate	Percentage of bacterial isolates
<i>E. coli</i>	15	60%
<i>E. asburiae</i>	2	8%
<i>P. aeruginosa</i>	2	8%
<i>K. pneumoniae subsp. pneumoniae</i>	1	4%
Total	20	80%

Table.2 Antibiotic susceptibility pattern of *E. coli* isolated from diarrheic pigs faecal samples

Antibiotics	Sensitive (%)	Moderate sensitive (%)	Resistant (%)
Ampicillin/Sulbactam	15 (100%)	-	-
Amoxicillin clavulanate	1 (6.66%)	6 (40%)	8 (53.33%)
Cefoperazone	4 (26.66%)	5 (33.33%)	6 (40%)
Cefotaxime	3 (20%)	4(26.66%)	8 (53.33%)
Chloramphenicol	15 (100%)	-	-
Ciprofloxacin	9 (60%)	2 (13.33%)	4 (26.66%)
Co-trimoxazole	5 (33.33%)	1 (6.66%)	9 (60%)
Gentamicin	15 (100%)	-	-
Kanamycin	9 (60%)	4 (26.66%)	2 (13.33%)
Levofloxacin	10 (66.66%)	2 (13.33%)	3 (20%)
Moxifloxacin	8 (53.33%)	1 (6.66%)	6 (40%)
Tetracycline	7 (46.66%)	3 (20%)	5 (33.33%)

Fig.1 Isolation and characterization of bacterial isolates (A) Lactose fermenter colonies on MLA (B) Lactose non-fermenter colonies on MLA (C) Gram's staining showing gram negative *E. coli* cells (D) Gram's staining showing gram negative lactose non-fermenter *Pseudomonas aeruginosa* (E) *E. coli* colonies with green metallic sheen on EMB agar (F) Pink colonies of *P. aeruginosa* on BGA

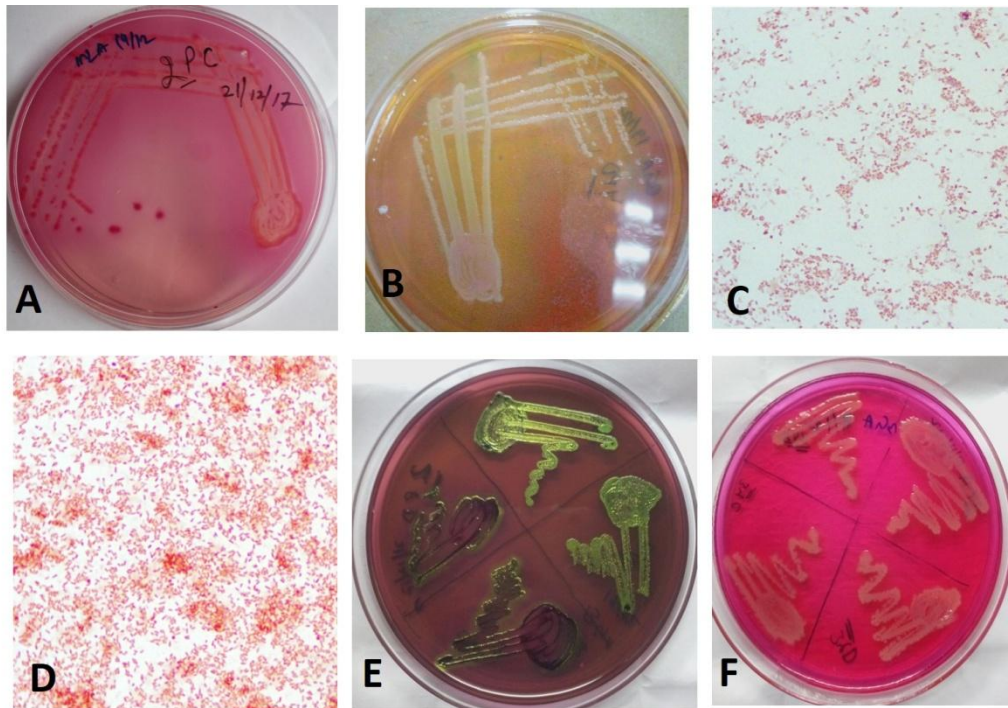
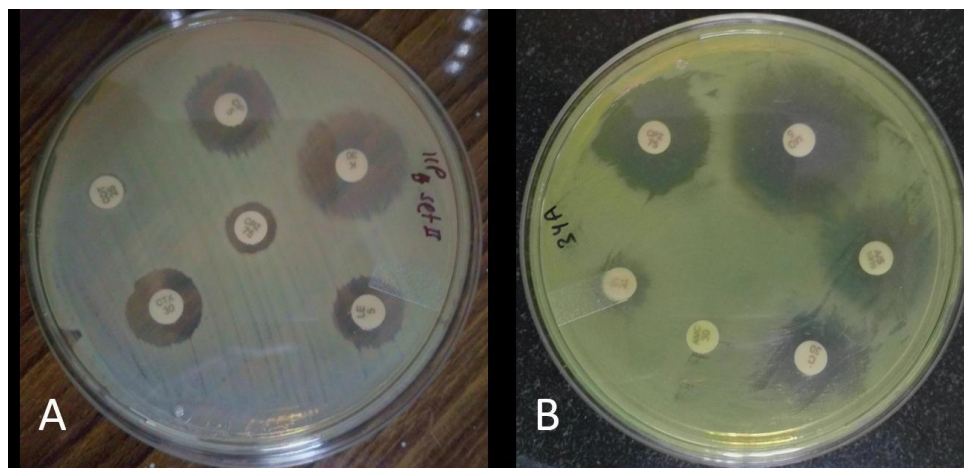


Fig.2 Antibiotic susceptibility testing using disc diffusion method (A) *E. coli* (B) *P. aeruginosa*. The antibiotics used were amoxicillin clavulanate (20/10 mcg), ampicillin/sulbactam (10/10 mcg), cefoperazone (75 mcg), cefotaxime (30 mcg), chloramphenicol (30 mcg), ciprofloxacin (5 mcg), cotrimoxazole (25 mcg), gentamicin (10 mcg), kanamycin (30 mcg), levofloxacin (5 mcg), moxifloxacin (5 mcg) and tetracycline (30 mcg)



The results showed that all five isolates were 100% sensitive to gentamicin, ampicillin/sulbactam and cefoperazone. The *P. aeruginosa* isolates were 100% sensitive to moxifloxacin, ciprofloxacin, levofloxacin whereas *E. asburiae* isolates were 100% sensitive to ciprofloxacin, cefotaxime, cefoperazone, gentamicin and ampicillin/sulbactam. The only isolate of *K. pneumoniae pneumoniae* was found to be sensitive to chloramphenicol, gentamicin, ampicillin/sulbactam and levofloxacin. All three (*P. aeruginosa*, *E. asburiae* and *K. pneumoniae pneumoniae*) bacteria were completely resistant to amoxicillin clavulanate and cotrimoxazole. The presence of antibiotic resistance genes primarily among *E. coli* isolates explains why some diarrheic cases were refractory to antibiotic therapies.

Escherichia coli is a Gram-negative bacteria of family Enterobacteriaceae and the causal and associated agent of various diseases of pigs, including neonatal diarrhea and PWD, which are responsible for death in suckling and weaned pigs, respectively worldwide (Fairbrother *et al.*, 2012). Depending upon the severity of the disease, the cost of PWD was estimated to vary from €40 to €314 per sow (Sjölund *et al.*, 2014), which is equivalent to INR 3200-25250 per sow. This is in addition to the heavy losses in piglets due to decreased weight gain, cost of treatment, vaccination, use of feed supplements and mortality (Fairbrother *et al.*, 2012). In the present study, the pig owners have informed us that they do not use antibiotics as feed additives and hence the presence of antibiotic resistant strains of bacteria in piglets might be due to non-prudent use of antibiotics by unqualified people in that area. During the outbreaks of PWD, quick actions are required and hence the antibiotic treatment is prescribed well before the results of sensitivity testing. Therefore, it is the responsibility of practitioner to choose effective antibiotics (Luppi, 2017).

To maintain a profitable porcine industry, it is very essential to diagnose the etiological agent of PWD. In case of bacterial involvement, the assessment of antibiotic susceptibility pattern for timely treatment, to reduce the unnecessary cost and development of antibiotic resistance is must. As the organisms are acquiring resistance against commonly used antibiotics, determination of antimicrobial sensitivity test becomes necessary, so that non-prudent use of antibiotics and development of resistance can be prevented. This would also help in preventing the spillover of the antibiotic resistant strains to other livestock and human population.

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