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Research Article

COMPARATIVE STUDY ON BIOFILM FORMATION AMONG DIFFERENT *ESCHERICHIA COLI* SEROTYPES ISOLATED FROM DIARRHEIC PIGLETS

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ABSTRACT

Biofilms provide an environment for transfer of plasmid carrying antibiotics resistance genes among bacteria and thus rendering antibiotics ineffective against such bacteria. The present study was aimed to characterize previously isolated 14 *E. coli* isolates by serotyping and biofilm formation ability. The biofilm formation ability was detected using qualitative and quantitative method. The serotyping results distributed maximum isolates to serotype O83 (5/14) followed by O120 (2/14), O126 (2/14) and one each as O8 and O20 by agglutination. Out of these, three *E. coli* isolates remained untypable. The qualitative tube adherence and Congo red agar binding methods revealed nine and four isolates as biofilm producers respectively. On comparing the findings of qualitative methods with quantitative tissue culture plate method employing two different media, it was observed that three isolates each were strong biofilm producers and three each as moderate biofilm producer, respectively in 1/20 diluted tryptone soy broth (nutrient depleted media) and tryptone soy broth (nutrient rich media). Of serotypes, serotype O83 formed moderate to strong biofilms with tissue culture plate method. The current study generated important epidemiological data for further exploration of role and mechanism of biofilm producing *E. coli* in piglet diarrhea.

Keywords: Biofilms, *Escherichia coli*, Piglet diarrhea, Serotype O83, Tissue culture plate method

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The enteric infections in piglets, particularly post weaning diarrhea (PWD) caused by enterotoxigenic *E. coli* (ETEC) is a huge economic problem to pig industry worldwide. The cost includes reduced weight gain, cost of antibiotic treatment, mortality and use of feed supplements (Mittal *et al.*, 2018). Biofilm is an assemblage of irreversibly attached microbial cells which starts with the microbial adhesion with a subsequent production and accumulation of an extracellular matrix composed by one or more polymeric substances such as proteins, polysaccharides, humic substances, extracellular DNA and sometimes other signaling molecules (Skyberg *et al.*, 2007). The bacterium in biofilms enjoys various benefits such as transfer of virulence and antibiotic resistance gene which makes the bacteria in biofilms more resistant to host defences, and antibiotics in comparison to planktonic cell (Rodriguez *et al.*, 2019).

E. coli biofilms are implicated in persistent infections of humans and animals (Skyberg *et al.*, 2007). Various *E. coli* strains produce amyloid fibers called as curli that serve as a structural scaffold to promote biofilm assembly and other community behaviors (Rodriguez *et al.*, 2019). The curli fibers are expressed to form characteristic crystalline black colored colonies when bind to congo red dye which enables the identification of curli producing strains of bacteria. There are several methods available for detection and quantification of biofilms. Qualitative methods such as tube method and Congo red agar test and quantitative as tissue culture plate methods have been previously used to detect biofilm formation by bacterial isolates.

Serotyping has been used previously to assess the epidemiology and pathogenicity of *E. coli* isolates as high correlation between enteric pathogens and serogroups have been reported (Stenutz *et al.*, 2006). The most common *E. coli* serotypes previously associated with piglet diarrhea worldwide include O8, O20, O120, O138, O64, O9, O101, O147, O149 and O157 (Mandakini *et al.*, 2015). In Indian context the serotypes isolated from diarrheic piglets includes O8, O20, O26, O147, O120, O5, O60, O51, O13 and untypable strains (Mandakini *et al.*, 2015; Samanta *et al.*, 2015). Very less data is available related to *E. coli* serogroups prevalent in PWD in piglets and far more scarce data associated to biofilm production ability is available in India.

So, this study was undertaken to identify the *E. coli* serotypes associated with post weaning diarrhea in piglets and their biofilm formation ability. The present study also compared the three available methods for biofilm detection, to propose most reliable and suitable method amongst them.

MATERIAL AND METHODS

Previously isolated and characterized *E. coli* isolates (n=14) from post weaned diarrheic piglets were used in the study. These isolates were previously confirmed as *E. coli* using conventional PCR (uspA gene) and Vitek 2 Compact. These isolates were also studied for antimicrobial

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susceptibility, antimicrobial resistance genes and presence of ESBL using phenotypic as well as conventional PCR (Mittal *et al.*, 2018; Grakh *et al.*, 2021). Stock cultures maintained in 50% glycerol were revived by seeding on MacConkey's lactose agar. The *E. coli* isolates were then processed for serotyping and biofilm formation ability. The ATCC 25922 was used as a positive control for biofilm formation in all the methods (Grakh, 2019).

Serotyping

The pure cultures of *E. coli* isolates in nutrient agar slants were submitted to National Salmonella and Escherichia Centre, Central research Institute, Kasauli, Himachal Pradesh, India for confirmation and serotyping by agglutination test.

Congo red agar test

All the isolates were subjected to Congo red dye binding test by streaking the *E. coli* isolates and ATCC 25922 on Congo red agar, as per the method described by Freeman *et al.* (1989).

Tube method

Tube method was done according to assay described by Christensen *et al.* (1985) using Trypticase soya broth with 1% glucose.

Tissue culture plate method

Biofilm formation and its quantification was performed in 96-well polystyrene microtiter plates as described previously (Skyberg *et al.*, 2007). Briefly, the *E. coli* cultures were inoculated in 5ml of Luria-Bertani (LB) broth and incubated overnight at 37 °C. Broth was adjusted to OD 600 = 0.05 (4×107 cells/ml) by diluting the overnight culture in tryptone soy broth (TSB) and 1/20 TSB. A volume of 200 µl (8×106 cells) aliquots of the dilution were then dispensed into microtiter plate wells in triplicate. Uninoculated TSB and 1/20 TSB were used as negative control and ATCC 25922 was used as a positive control for biofilm. The contents of the plates were gently poured off and the plates were washed with double distilled water and then stained with 0.1% crystal violet for 30 min. The plates were then washed again, air dried and adherent cells were resolubilized with 80: 20 solution of ethanol and acetone. A volume of 150 µl of this solution was then transferred to a new microtiter plate, and the optical density (OD) of each well was measured at 570 nm (OD570) using an automated micro plate reader. All tests were carried out in triplicate, and the average value of results was taken.

Based on the OD produced by bacterial biofilms, bacterial cultures were classified into weak, moderate, strong, and non-biofilm producers the following categories as described by Grakh, 2019. The cutoff OD (ODc) was calculated as ODc= average OD of negative control + 3 (SD of negative control) where SD= Standard deviation.

RESULTS AND DISCUSSION

Serotyping

Maximum isolates (5/14) were found to be of serotype O83 followed by O120 (2/14), O126 (2/14), and one isolate of serotype O8 and O20 each. Three isolates were found to be untypable and were not assigned to any serogroup. A large number of serotypes of *E. coli* have been described, but only limited numbers of them have been associated with enteric infections in pigs as these serotypes are not commonly found in *E. coli* strains isolated from the normal gut (Mandakini *et al.*, 2015). Serogroup O8, O20 and O120 found in present study have been commonly reported from post weaning diarrheic piglets in India and worldwide (Mandakini *et al.*, 2015), whereas the O8 and O20 serotype are most common ETEC strains responsible for PWD (Mandakini *et al.*, 2015). The serogroup O8 as reported in current study were also earlier associated with piglet oedema, a disease caused by pathogenic *E. coli* (Garabal *et al.*, 1996). Similarly, serogroup O126 was associated with enteropathogenic *E. coli* (EPEC) which causes diarrhea in children worldwide (Campos *et al.*, 2004). The occurrence of O126 in piglet diarrhea raises some serious public health and zoonosis concerns. The serogroup O83 with maximum occurrence in the present study have never been reported from diarrheic piglets before. However, O83 has been previously reported in India from diarrheic goat kids in Gujarat and as major pathogenic serotype in humans responsible for urinary tract infections characterized as urinary pathogenic *E. coli* (UPEC) (Sharma *et al.*, 2016). In rural India, pigs are kept loose to feed on human and animal excreta (feces and urine) and might serve as a vehicle for transmission of O83 *E. coli* to pigs (Socioeconomic and caste census, 2015). It is likely possible that the pig owners also rear goats to supplement their income or as a source of milk, which might be associated for such transmission. However, more work needs to be done to ascertain such epidemiological links. Three isolates which remained untypable might be important as various other virulence factors and antigens might be responsible for pathogenesis of piglet diarrhea. Belonging to a specific serotype does not confer virulence on bacteria, but serotyping can be used as an epidemiological probe for pathogenicity, as there is a high positive correlation between certain serotypes and enteric pathogenicity (Stenutz *et al.*, 2006).

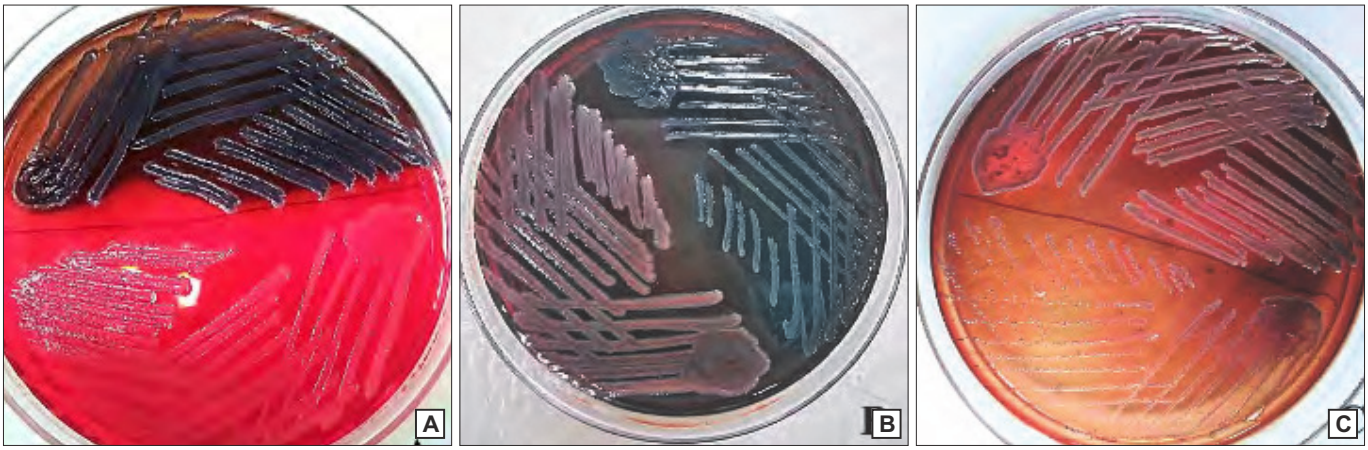

Fig. 1. Biofilm formation of *E. coli* isolates on Congo red agar. Black crystalline colonies (A and B) are indicating strong biofilm/slime producers. Red, pink, and transparent colonies (A, B, C) are negative biofilm/slime producers.

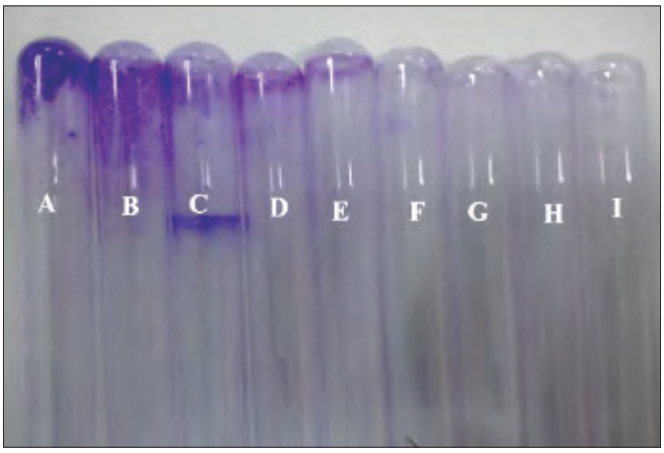

Fig. 2. Biofilm formation of *E. coli* isolates using Tube method. A; Positive control (ATCC 25922), B and C: Strong biofilm producers, D and E: Moderate biofilm producers, G: Weak biofilm producer, H: Negative control, F, and I: Non-biofilm producer

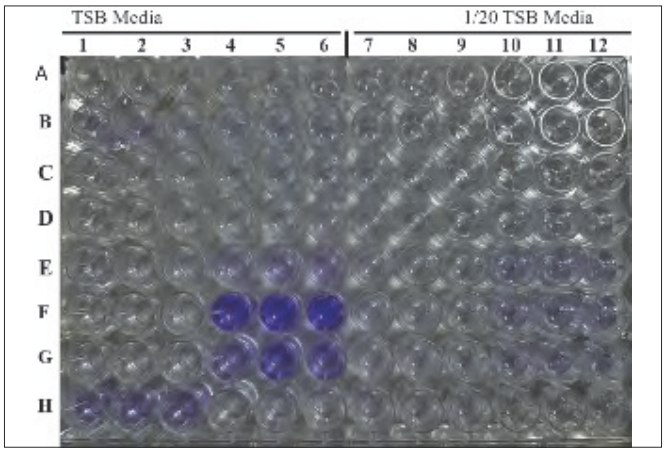

Fig. 3. Biofilm formation of *E. coli* isolates in 96 well microtiter plate using TSB and 1/20 TSB media.

Table 1. Biofilm producing *E. coli* isolates by different methods

S. No.	Method/Test used	Total biofilm producers (n=14)	Biofilm formation (n=14)			
			Strong	Moderate	Weak	No biofilm
1	Tube method	9	4	4	1	5
2	Congo red agar	4	4	-		10
3	Tissue culture plate using 1/20 TSB	9	3	3	3	5
4	Tissue culture plate using TSB	11	3	3	5	3

Biofilm Formation

Based on Congo red dye binding and characteristic crystalline black colonies four isolates were characterized as strong biofilm producers on Congo red agar (Fig. 1), whereas, pinkish to grayish colonies with black zone in the center were taken as negative or non-producers. The Tube method identified four isolates as strong biofilm producers when compared with the positive control by observing the staining of bottom and wall of test tubes (Table 1 and Fig. 2). The quantitative Tissue culture plate method revealed three isolates each, as strong and moderate biofilm producers with both media i.e., 1/20 TSB and TSB respectively (Table 1 and Fig. 3).

A1-A3 and A7-A9: Non biofilm producers, B1-B3, H1-H3, E4-E6, B7-B9, H7-H9 and E10-E12: Moderate biofilm producers, F4-F6 and F10-F12: Strong biofilm producers, G4-G6 and G10-G12: ATCC 25922 (Positive Control for strong biofilm) and H4-H6 and H10-H12: Negative control (Uninoculated media).

Biofilms in pig industry are responsible for reduced efficacy of disinfectants and antimicrobials, leading to persistence of bacteria such as *E. coli* in sheds and surrounding environment including water system (Tajbakhsh *et al.*, 2016). Studies by Santos *et al.*, 2018, characterized 46.03% (58/126) *E. coli* isolates from pigs as biofilm producers. Although the number of samples studied in current study are less and it is difficult to compare with other studies on this area, but still the

Table 2. Serotype distribution and biofilm formation of the isolates used in the study.

Sr. No.	Isolate number	Serotype	Biofilm type with			
			Tube method	Congo red agar test	Tissue culture plate (TSB)	Tissue culture plate (1/20 TSB)
1.	2Y	UT	Moderate	No biofilm	Weak	Weak
2.	4Y	O8	No biofilm	Strong	Moderate	Moderate
3.	5P	O120	No biofilm	No biofilm	No biofilm	No biofilm
4.	6P	O20	Moderate	No biofilm	Weak	Weak
5.	7P	O126	Strong	Strong	Weak	Weak
6.	8P	O120	No biofilm	No biofilm	No biofilm	No biofilm
7.	9P	O126	Strong	No biofilm	Moderate	Moderate
8.	10P	O83	Weak	No biofilm	Strong	Strong
9.	11PB	O83	Moderate	No biofilm	Weak	No biofilm
10.	12P	UT	No biofilm	Strong	Weak	No biofilm
11.	13P	UT	Strong	No biofilm	No biofilm	No biofilm
12.	14P	O83	Moderate	No biofilm	Moderate	Moderate
13.	16P	O83	Strong	Strong	Strong	Strong
14.	21P	O83	No biofilm	No biofilm	Strong	Strong
15.	ATCC 25922	-	Strong	Strong	Strong	Strong

*UT= Untypable

presence of moderate-strong biofilm producing isolates in pigs is an important finding of current study. The isolates used in the study were found to be multiple drug resistant and had ESBL as well as other AMR genes (Mittal *et al.*, 2018 and Grakh *et al.*, 2021), which might be due to biofilm forming ability of these isolates.

Curli expression by *E. coli* might be the initial step for biofilm formation, but many other factors are required for mature biofilm (Rodriguez *et al.*, 2019). As evident from Table 2, four strong biofilm producers on Congo red agar, also produced moderate to strong biofilms in Tissue culture plate method. Similarly, only one isolate of *E. coli* that produced strong biofilm in tube method produced strong biofilm in tissue culture plate method (Table 2), which might be due to the fact that glass (hydrophilic) is less favorable to colonization (Rodriguez *et al.*, 2019). The production and morphology of biofilms can also vary according to other factors such as pH, nutrient, source of origin of bacteria, test method employed for detection, media used and oxygen availability (Rodriguez *et al.*, 2019). Similar variation was also observed in the present study where variation in biofilm formation in 1/20 TSB and TSB media, might be due to presence of more nutrients in TSB as compared to diluted TSB (Reisner *et al.*, 2006; Rodriguez *et al.*, 2019). Except for one isolate belonging to serogroup O83, none of the isolate showed consensus in terms of strong biofilm producer across all three methods of biofilm formation. It is also observed that Congo red agar test and tube method are far less reliable for detection of biofilm producing isolates (Reisner *et al.*, 2006). The present study indicate that tissue culture plate is more reliable, economical, and quick method for biofilm detection than tube method and congo red agar test.

CONCLUSION

The biofilm formation ability of *E. coli* strains from diarrheic weaned piglets can be ascertained using tissue culture plate method. The present study found serotype O83 to be associated with diarrhea in pigs which was previously reported only as human urinary tract pathogen. Further studies on toxins and other virulence factors can be undertaken to reveal pathogenicity mechanisms of these *E. coli* strains in post weaning diarrhea in piglets.

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