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KG Chaitanya
M.V.Sc Student, Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Tirupati, Andhra Pradesh, India

T Madhava Rao
Registrar, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

A Jagadeesh Babu
Professor and Head, Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Tirupati, Andhra Pradesh, India

B Sreedevi
Professor and Head, Department of Veterinary Microbiology, College of Veterinary Science, Tirupati, Andhra Pradesh, India

Corresponding Author:
KG Chaitanya
M.V.Sc Student, Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Tirupati, Andhra Pradesh, India

Detection of biofilm forming ability of *Escherichia coli* isolates from pigs and pork samples

KG Chaitanya, T Madhava Rao, A Jagadeesh Babu and B Sreedevi

Abstract
Biofilms are the most common mode of bacterial growth in nature and are also important in clinical infections. The present study was conducted to find the prevalence of biofilm formation of *E. coli* isolated from pig fecal samples (250), pork (120) and water samples (60) used during pig farming and during slaughter, 165 *E. coli* isolates were obtained from 430 samples of which 132 isolates from pig fecal samples, 17 from pork and 16 isolates from water samples. All the 165 *E. coli* isolates were subjected to assessment of biofilm production by modified congo red agar plate (MCRA) /Slime production method and microtitre plate method. From the 165 *E. coli* isolates, 95 (57.57%) and 102 (61.81%) were biofilm producers in MCRA method and microtitre plate method respectively. In the present study, biofilm production in Microtiter plate method was slightly higher than slime production. Most of the isolates (96.07%) were low biofilm producers, whereas 3.92 percent of isolates were moderate biofilm producers.

Keywords: Biofilm, *E. coli*, modified Congo red agar plate method, microtitre plate method

1. Introduction
Bacterial biofilms are structured communities of bacterial cells enclosed in a self-produced polymer matrix (consisting of proteins, exopolysaccharide and nucleic acid) that is attached to biotic and abiotic surfaces. Biofilms protect and allow bacteria to survive and thrive in hostile environments as well as facilitate chronic/persistent infections. Bacteria within biofilms can withstand host immune responses, and are much less susceptible to antimicrobials and disinfectants when compared to their planktonic counterparts (Tremblay *et al*., 2014) [16]. *E. coli* biofilm development is a complex process that leads to beautiful structures that are important for disease and for engineering applications (Jayaraman *et al*., 1999) [5].
There are various methods used to detect biofilm production of *E. coli* like Microtitre plate method, Tube method (TM), Congo red agar method (CRA), Modified congo red agar method (MCRA), bioluminescent assay, piezoelectric sensors and fluorescent microscopic examination (Panda *et al*., 2016) [10]. In the present investigation MCRA and microtitre plate method are used to detect biofilm producing ability of *E. coli* isolates from Pigs, pork and water samples.

2. Materials and Methods
2.1 Sample collection
Pig fecal samples (250) from young and adult pigs including both diarrhoeic and non-diarrhoeic pigs, pork sample (120), water samples (60) used in pig farming and water samples were collected from AICRP on pigs, Tirupati and different villages in and around Tirupati town, A.P. All the samples were collected in suitable aseptic containers and were transported to laboratory under chilled conditions and processed within 24h of collection. *E. coli* obtained from these samples were analysed further for biofilm production by MCRA and Microtitre plate methods.

2.2 Modified Congo red agar method
All the *E. coli* isolates in this study were checked for their ability to produce biofilm using Modified Congo red agar of the following composition as described by Mariana *et al.* (2009) [7]. Congo Red Agar plates were inoculated with the *E. coli* isolates and incubated at 37°C for 48 hrs, and subsequently at room temperature for 2-4 days. The slime producing strains forms strong black pigmentation to slightly black colonies which indicates positive for biofilm production whereas non-slime producers develop red colonies which indicates negative for biofilm production (Fig 1).

2.3 Microtitre plate method

All the *E. coli* isolates in this study were checked for their ability to produce 53 biofilm using flat well 96 microtiter plate as procedure described by Bangar and Ballal (2008) [1]. Inoculated 200 µl of 0.45% glucose-rich MH broth in 96 well-bottom microtiter polystyrene plates with 5 µl of an overnight Luria broth culture grown at 37℃ with shaking. The sample was incubated overnight (18 hrs) at 37℃ and visualized by staining with 0.5% crystal violet for 5 min after washing with water. The biofilm was quantified after adding 200 µl of 95% of ethanol by Multimode reader at 570 nm (Fig 2). The interpretation of biofilm production was done according to the criteria of Stepanovic *et al.* (2007) [13] which is shown in Table-1. Optical density cut off value (ODc) was calculated by taking ODc = Mean optical density (OD) of negative control + 3X standard deviation (SD) of negative control. Isolates with optical density ≤ ODc were considered as non-biofilm producers. Isolates with OD ≤ 2 X ODc were interpreted as weak biofilm producers, isolates with OD between 2 to 4 X ODc were taken as moderate producers of biofilm and those isolates with OD > 4 X ODc were considered as strong biofilm producers.

ODs of negative controls- 0.04, 0.008, 0.019, 0.012, 0.049, 0.015.

Mean ODs of negative controls = 0.024

1SD = 0.017, 3SD = 0.051 (ODc = Mean + 3SD, 0.024 + 0.051 = 0.075)

ODc = 0.075

Table 1: Interpretation of biofilm production

Average OD value		Biofilm production
ODs ≤ ODc	≤ 0.075	None
ODs ≤ 2 X ODc	< 0.15	Weak
ODs 2 X ODc ≤ 4 X ODc	0.15 – 0.3	Moderate
ODs > 4 X ODc	> 0.3	Strong

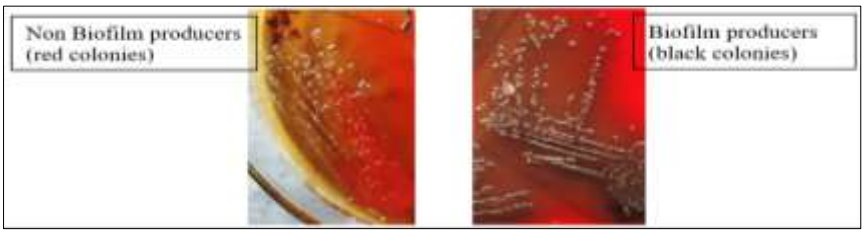

Fig 1: Modified Congo red agar plate showing biofilm formation

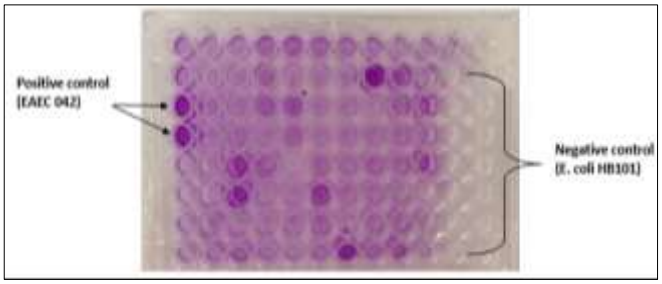

Fig 2: Biofilm formation on a polystyrene microtiter plate. The blue wells are biofilm positive

3. Results

In most of the studies, it was found that the MCRA could not differentiate between strong, moderate and weak biofilm producers (Panda *et al.*, 2016) [10]. In the present study, isolates producing black colonies with dry crystalline consistency are considered as biofilm producers. Out of 165 *E. coli* isolates tested, 95 (57.57%) were found to produce black colonies within 24-48 hours. The remaining 70 (42.42%) *E. coli* isolates failed to produce black colonies even after 72 hrs of incubation. Out of 95 slime producers, 69 (72.63%) were from pig fecal samples, 16 (16.84%) were from pork and the remaining 10 (10.52%) were from water samples.

On performing quantitative biofilm assay all the *E. coli* isolates were classified according to Stepanovic *et al.* (2007) [13] into four major categories as high biofilm producers, moderate biofilm producers, weak biofilm producers and non-biofilm producers. The criteria were adopted based on Optical density (OD) values at 570 nm wavelength, by considering both positive and negative control OD values. Thus, in the present study those *E. coli* isolates showing an average values of 0.3 and above (OD 570 nm) were considered as high biofilm producers, isolates which have shown OD values from 0.15 to 0.3 (OD 570 nm) were described as moderate biofilm producers, 0.15 and below (OD 570 nm) were considered as low biofilm producers and the isolates which 70 have shown OD values of 0.075 and below were considered as non-biofilm producers. The results of this study was shown in Table 2.

Table 2: Biofilm production of *E. coli* isolates from different samples

Biofilm type	No. of samples			
	Pig fecal samples		Pork samples	Water samples
	Young	Adult		
Strong	4	7	5	1
Moderate	10	18	11	5
Weak	17	20	0	6
Total	31	45	16	12
Non-biofilm producers	26	30	0	5

Out of 165 *E. coli* isolates, 102 (61.81%) were positive for biofilm production**.** 70 (42.42%) of slime negative isolates were also negative for biofilm production whereas the 7 (4.24%) slime positive isolates were found to be negative for biofilm production. None of the isolates were high biofilm producers.

Out of 132 *E. coli* isolates obtained from pig fecal samples, 77 (58.33%) were positive for biofilm production and all of them were low biofilm producers. Among 77 biofilm producing isolates, 30 (38.96%) were from young pigs and 47 (61.03%) were from adult pigs. Out of 16 *E. coli* isolates obtained from pork samples, all the isolates were biofilm producers. Among the 16 isolates, 4 (25%) isolates have shown moderate biofilm production whereas 12 (75%) isolates were low biofilm producers. Out of 17 *E. coli* isolates obtained from water samples, 9 (52.94%) isolates were low biofilm producers.

4. Discussion

Out of 165 *E. coli* isolates 95 (57.57%) and 102 (61.81%) isolates were positive for biofilm production by Modified Congo red agar (Slime production) and Microtiter plate method respectively. The failure of seven slime positive isolates to produce biofilm in-vitro can be attributed to the variation in phenotypic expression of biofilm and/or slime production. Such observations were also made by Dadawala *et al.* (2010) [2] where they found that out of 14 *E. coli* isolates tested, 12 isolates were slime positive and 10 isolates produced biofilm on microtiter plate.

In the present study, biofilm production in Microtiter plate method slightly higher than slime production. This is in concordance with studies by Mathur *et al.* (2006) [8], Hassan *et al.* (2011) [4] and Panda *et al.* (2016) [10] where Microtiter plate method detected 47.3%, 63.6% and 45.6% of biofilms followed by 5.2%, 10% and 11% by Slime production on CRA plate. Whereas Tayal *et al.* (2015) [15] and Dhanalakshmi *et al*. (2018) [3] reported that Slime production in CRA plate (40.8% and 46.97% respectively) detected more biofilm producers than Microtiter plate method (27% and 39.77% respectively). The variations observed in Slime production in CRA plate might be due to the fact that different studies used modifications in the media with greater sugar content and few studies also had increased the duration of incubation promoting greater biofilm production by bacteria (Dhanalakshmi *et al*., 2018) [3]. The variations observed in Microtiter plate method might be because of the differences in the sources from which the strains were isolated and differences in the methodology employed in the study (Dhanalakshmi *et al*., 2018) [3].

The present findings were in agreement with Risal *et al*. (2018) [11], Sharma *et al.* (2009) [12], Niveditha *et al.* (2012) [9] and Tajbakhsh *et al.* (2016) [14] who found 64%, 67.5% and 60% and 61.5% of *E. coli* isolates were biofilm producers. While lower percent compared to the present findings was observed by Dhanalakshmi *et al.* (2018) [3] who found only 42% of *E. coli* were biofilm producers. The variations in the *E. coli* isolates to produce biofilm is associated with variations in the expression of a variety of virulence factors like adhesins (Niveditha *et al.*, 2012) [9].

Based on the Stepanovic *et al.* (2007) [13] criterion for interpretation of biofilm production of *E. coli*, in the present study all the biofilm positive *E. coli* isolates from pigs (58.33%, 77/132), water samples (52.94%, 9/17) from pig rearing and during slaughter were low biofilm producers. Majority of the pork isolates (75%) were low biofilm producers where as 25% of the isolates were moderate biofilm producers. These findings were in agreement with Vijay *et al.* (2015) [17] and Manjushree *et al.* (2019) [6] who reported that majority of the animal isolates and environmental isolates (water, soil) were low biofilm producers.

5. Conclusion

In the present study, biofilm production in Microtiter plate method was slightly higher than slime production. Most of the isolates (96.07%) were low biofilm producers, whereas 3.92 percent of isolates were moderate biofilm producers.

6. References

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