RESEARCH ARTICLE

Evaluation of Biofilm Formation Capacity of *Pasteurella multocida* and its Relationship with Antibiotic Resistance

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Abstract

Pasteurella multocida is a Gram-negative bacterium that causes haemorrhagic septicaemia in cattle and buffaloes. These organisms are involved in the formation of biofilm and can evade treatment. There is no definitive study to screen the genes associated with biofilm production associated with *Pasteurella multocida*. The present study deals with the real-time PCR based approach for screening of genes associated with biofilm formation. Out of 10 isolates screened for biofilm formation, five of them produced biofilm on Congo red agar of which all are resistant to major antibiotics especially cotrimoxazole, nalidixic acid, enrofloxacin and tetracyclines. All the isolates show presence of genes associated with biofilm formation indicating that other factors influenced the biofilm production. We suggest that the future studies may be targeted to unravel the other factors that influence the biofilm production.

Keywords: Biofilm, Haemorrhagic Septicaemia; *Pasteurella multocida*, Real-time PCR, tad locus genes.

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Introduction

Pasteurella multocida is an important opportunistic pathogen responsible for major animal diseases in both developed and developing countries. The major diseases of economic significance include haemorrhagic septicaemia (HS) in cattle and buffaloes, bronchopneumonia in sheep and goat, atrophic rhinitis in pigs, fowl cholera in poultry and snuffles in rabbits. In India**,** HS is the second most reported disease in buffaloes and cattle (Annual Report, 2020-21).

The pathogenicity of *P. multocida* is associated with various virulence factors such as capsule, lipopolysaccharide (LPS), outer membrane proteins, adhesion proteins, iron acquisition proteins, sialidases and dermonecrotic toxin. These virulence factors help in invasion of the host, avoid host defence mechanisms, injury to host tissues, and stimulate host inflammatory response (Harper *et al.,* 2006). Apart from these, formation of biofilm can be another important virulence factor for the treatment failure, development of antibiotic resistance and establishment of chronic carrier status (Rajagopal *et al.,* 2013; Chakraborty *et al.,* 2018; Prajapati *et al.,* 2020). Biofilm formation is a process whereby microorganisms irreversibly attach to and grow on a surface and produce extracellular polymers that facilitate attachment and matrix formation, resulting in an alteration in the phenotype of the organisms with respect to growth rate and gene transcription. The mechanism of biofilm formation including initial attachment, matrix formation, development and maturation in *P. multocida* is not well understood, and only a limited number of studies have been carried out on the virulence genes involved in biofilm formation (Emery *et al.,* 2017). Since the pathogenic behaviour and biofilm formation could be predicted by these genes, evaluation of these genes in *P. multocida* is necessary as it is an important respiratory pathogen. Because these genes are regarded as necessary factors for intercellular adhesion, it could be assumed that these genes are important for the formation of the bacterial multilayer in biofilm production (Jabeen *et al.,* 2019). Hence,

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the present study has been taken up with an objective to detect biofilm formation associated genes in vaccine strain and filed isolates of *P. multocida*

Materials and Methods

Ethical Statement

This study was conducted with prior permission and in accordance with the guidelines of PV Narasimha Rao Telangana Veterinary University, Rajendranagar, Hyderabad, Telangana.

Bacterial Strains

A total of 10 strains of *P. multocida* maintained at Department of Veterinary Microbiology, College of Veterinary Science, Rajendranagar were used for the study. These organisms were isolated from buffaloes that died of Haemorrhagic Septicaemia during the period from December 2020 to July 2021 from Andhra Pradesh state. Isolation was done according to the methods described by Quinn *et al.* (1994). *P. multocida* vaccine strain (P_{52} strain) procured from Telangana State Veterinary Biologicals & Research Institute, Hyderabad, Department of Animal Husbandry, Government of Telangana was used for comparison studies.

Evaluation for Antibiogram and Biofilm Formation:

The isolates (n=10) were cultured on 5% sheep blood agar and were subjected to Antibiotic sensitivity test by disc diffusion method (Bauer *et al.,* 1966) using following 21 antibiotic discs: Amoxycillin / Sulbactum (AMS 30/15 µg/disc), Amoxyclav (Amoxicillin/ Clavulanic acid) (AMC 20/10 µg/disc), Ampicillin (AMP 10 µg/disc), Ceftriaxone (CTR 30 µg/disc), Cefoperazone / Sulbactum (CFS 75/30 µg/disc), Ceftiofur (EFT 30 µg/disc), Cloxacillin (COX 30 µg/disc), Enrofloxacin (EX 5 µg/disc), Erythromycin (E 15 µg/disc), Gentamicin (GEN 10 µg/disc), Kanamycin (K 30 µg/disc), Lincomycin (L 15 µg/disc), Methicillin (MET 5 µg/disc), Tetracycline (TE 10 µg/disc), Cefotaxime (CTX 5 µg/disc), Ciprofloxacin (CIP 5 µg/disc), Co-Trimoxazole (Trimethoprim/ Sulphamethoxazole) (COT 25 µg/disc), Levofloxacin (LE 5 µg/disc), Nalidixic Acid (NA 30 µg/disc), Penicillin G (P 1 Unit), Tetracycline (TE 30 µg/disc) (HiMedia, Mumbai, India). The zone of inhibition around each disc was measured and the results were interpreted according to the CLSI guidelines (CLSI, 2010). The biofilm formation by *P. multocida* is evaluated *in vitro* upon culturing onto 0.08% Congo-Red agar (CRA) having 5% sucrose.

Analysis of Biofilm Forming Genes by Real-Time PCR:

The genomic DNA was extracted by phenol chloroform isoamyl alcohol (PCI) method as described by Sambrook and Russell (2006). Concentration of DNA was estimated by using Nanodroplite spectrophotometer (Thermoscientific). All the DNA samples were analysed by PCR using species-specific (PM-PCR) and type B-specific primers for confirmation of *P. multocida* and capsular type (Townsend *et al.,* 1998).

The real-time PCR was performed by targeting the four genes associated with biofilm formation and tissue colonization viz., *flp*D, *rcp*A, *tad*B and *pil*B(*hol*B) (Jabeen *et al.,* 2019. The primers were designed to the target genes using the whole genome sequence of our isolate (unpublished data). Briefly, the genome sequence was compared with 10 other reference strains from GenBank of NCBI in MEGAX software by multiple sequence alignment using clustal W algorithm and the consensus regions were selected for primer designing. The primers were designed using Primer Select program of DNASTAR package. The best primers with minimal dimers formation were chosen and the quality parameters were revalidated using oligo analyser. The primers were synthesized by Bioserve Biotechnologies (India) Pvt Ltd, Hyderabad. The details of four primer sets designed in this study are given in table 1.

Table 1: Primers designed and used in the study

Gene	Protein	Primer sequence (5'-3')	Product length
*rcp*A	Secretin	FP: TCTTGTGGGTGGAGAGCTAC RP: GGATTGCCCCAACTCCTTCA	191 bp
*flp*D	Pilus assemble protein	FP: TGTTTGGTGCTGTGTTGTAGG RP: ACTGTATGACGCACAAGTGG	156 bp
*tad*B	MaoC protein	FP: AGCGATTGGTGAAGAGCCTA RP: TGTCTGCAATCACTCTCCCT	158 bp
*pil*B (*hol*B)	Pilin	FP: CCTCTGCCCTTACTCGCTTA RP: TACACGGCAATCACAACGAC	113 bp

The Real time PCR assay was performed using DNA as a template and the assay was performed in Step One Plus (Applied Biosystems) real-time PCR machine. Briefly, each reaction was set to a total volume of 10 µL containing 1 µL of DNA template @ 20 ng/reaction, 0.5 µL each of forward and reverse primer @ 10 pmoles/reaction, 2.8 µL of nuclease free water and 5 µL of TB green Extaq II (Takara) at 1X final concentration along with 0.2 µL of Rox. The reaction was set for vaccine strain and 10 other isolates from current study individually using four sets of primers and each reaction was performed in triplicate. The PCR conditions were set at 96°C for 10 min followed by 40 cycles of 96°C for 30 s, 52°C for 30 s, 60°C for 30 s. The melt curve analysis was performed at the end of the reaction; starting from 50°C to 95°C with ramp speed of 0.3°C rise. The quality parameters were analysed based on the melt curve and standard deviation between the replicates. The Ct values were obtained from amplification plot and were adjusted according to the negative control.

The multiplex PCR was performed for the three genes *rcp*A, *pil*B and *tad*B using the same primers as listed for real-time PCR. Briefly, the reaction set for 50 µL consists of 200 ng of DNA template/reaction, 3 sets of forward and reverse primer @ 5 pmoles/reaction, Emerald Taq at 1x final concentration. The PCR conditions were set for initial denaturation of 96°C for 10 min followed by 40 cycles of 96°C for 30 s, 52°C for 30 s, 72°C for 30 s and a final extension of 72°C for 7 min. The samples were run on 2% agarose gel and

the gel was documented using Syngene gel documentation system.

Results and Discussion

After PM-PCR and capsular type B specific PCR, all the 10 isolates yielded an expected amplicons of 460 bp and 620 bp size respectively similar to the standard reference *P. multocida* (P_{52}) vaccine strain thus confirming isolates as *P. multocida*.

Biofilm formation may be a reason for antibiotic resistance by P. multocida:

There are only limited reports on biofilm production by *P. multocida* and the relevance to antibiotic resistance (Chakraborty *et al.,* 2018; Prajapati *et al.,* 2020). In this study, 21 antibiotics were used to establish antibiogram. Table 2 depicts the antibiotic resistance pattern for 10 isolates tested in the study. Further, these samples were tested for biofilm formation of which five isolates produced weak biofilm. No biofilm formation was observed in P_{52} vaccine strain. All the 5 isolates producing biofilm were shown resistance to at least four of the antibiotics. Prajapati *et al.* (2020) reported that the weak biofilm producers conferred resistance to erythromycin, whereas the strong biofilm producers conferred resistance to enrofloxacin and azithromycin. Here in this study, we report that the weak biofilm producers were conferring resistance to cotrimoxazole, nalidixic acid, tetracycline and enrofloxacin.

Table 2: Antibiotic Resistance pattern of the isolates under study

S.No	Name of the Antibiotic	Isolate No.									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	10
1	Amoxycillin / Sulbactum (AMS 30/15)	S	S	S	S	S	S	S	S	S	S
2	Amoxyclav (Amoxicillin/ Clavulanic acid) (AMC 20/10)	S	S	S	S	S	S	S	S	S	S
3	Ampicillin (AMP 10)	S	S	S	S	S	R	R	R	S	S
4	Ceftriaxone (CTR 30)	S	S	S	S	S	S	S	S	S	S
5	Cefoperazone / Sulbactum (CFS 75/30)	S	S	S	S	S	S	S	S	S	S
6	Ceftiofur (EFT 30)	S	S	S	S	S	S	S	S	S	S
7	Cloxacillin (COX 30)	S	S	S	S	S	S	S	S	S	S
8	Enrofloxacin (EX 5)	R	R	S	S	S	R	S	R	S	S
9	Erythromycin (E 15)	S	S	R	R	R	S	S	R	S	S
10	Gentamicin (GEN 10)	S	R	R	S	R	R	R	S	S	S
11	Kanamycin (K 30)	S	S	S	S	S	S	S	S	S	S
12	Lincomycin (L 15)	R	R	R	S	S	R	R	S	S	S
13	Methicillin (MET 5)	S	S	S	R	R	R	R	S	S	S
14	Tetracycline (TE 10)	R	R	R	R	R	R	R	S	S	S
15	Cefotaxime (CTX 5)	S	S	S	S	S	R	S	R	S	S
16	Ciprofloxacin (CIP 5)	S	S	S	S	S	S	R	R	S	S
17	Co-Trimoxazole (Trimethoprim/ Sulphamethoxazole) (COT 25)	S	R	R	S	R	R	R	S	S	S
18	Levofloxacin (LE 5)	S	S	S	S	S	S	S	S	S	S
19	Nalidixic Acid (NA 30)	R	S	S	S	S	R	R	R	S	S

(Table continued)

(Table continued)

S.No	Name of the Antibiotic	Isolate No.									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	10
20	Penicillin G (P 1 Unit)	S	S	S	S	S	R	S	R	S	S
21	Tetracycline (TE 30)	S	S	S	S	S	S	S	S	S	S
Biofilm formation by CRA method		-	+	+	-	-	+	+	+	-	-
PCR Result	*tad*B	+	+	+	+	+	+	+	+	+	+
	*pil*B	+	+	+	+	+	+	+	+	+	+
	*rcp*A	+	+	+	+	+	+	+	+	+	+
	flpD	+	+	+	+	+	+	+	+	+	+

Production of intercellular adhesion molecules by tad locus and other genes like *fim*A, *hsf*1, *hsf*2, *pfh*A, *ptf*A, and *pil*B are responsible for biofilm formation in many members of the Pasteurellaceae family (Jabeen *et al.,* 2019). The tad locus was reported to have 14 genes (*flp*1-*flp*2-*tad*V-*rcp*CAB-*tad*ZABCDEFG) involved in biofilm formation (Jabeen *et al.,* 2019). The tad locus was also reported in many other Gram-negative organisms like Pseudomonas (Rashid *et al.,* 2000), Salmonella (Agarwal *et al.,* 2011), *E. coli* (Olson *et al*., 2002) and Neisseria (Zambori *et al.,* 2013) and was responsible for biofilm formation. However, there are only limited reports on the role of these genes in biofilm formation during infection (Moraes *et al.,* 2014; Jabeen *et al*., 2019). *P. multocida* was reported phenotypically as a weak biofilm producer (Emery *et al.,* 2017; Prajapati *et al.,* 2020).

Screening of genes associated with biofilm production by real-time PCR:

The tad locus along with other adhesion genes like *pil*B play an important role in biofilm formation. Hence, the four genes (*tad*B, *pil*B, *rcp*A, *flp*D) were targeted by real-time PCR in this study. Real-time PCR is the most sensitive and reliable method of detection of genes (Jyoti Kumar *et al.,* 2020). All the 10 isolates screened along with the vaccine control tested positive with a Ct value ranging from 11 to 16. Fig.1 is the representative amplification curve showing Ct values. The standard deviation between the replicates was found to be in the range of 0.1-0.3 that indicates the assay is more reliable. Further, there was only a single clear peak of dissociation in the melt curve indicating the assay is more specific (Fig. 2). The samples were further analysed on 2% agarose gel and found a single amplicon of size 113 bp, 156 bp, 158 bp and 191 bp for *pil*B *(hol*B), *flp*D, *tad*B and *rcp*A respectively (Fig. 3).

To our knowledge, this is the first study to use real-time PCR assay to analyse the genes associated with biofilm production in *P. multocida*. Of the 10 isolates screened in the study, the genes associated with biofilm production were found in all isolates and vaccine strain, but only 5 isolates are producing weak biofilm on Congo red agar. However, previously it was reported that though the biofilm producing genes were present in *P. multocida* isolates they may be either weak biofilm producers or non-biofilm producers (Emery *et al.,* 2017; Moraes *et al.,* 2014). Petruzzi *et al.* (2017) reported that, capsular polysaccharide interferes with biofilm formation by *P. multocida*. Beloin and Ghigo (2005) opined that, several genetic factors participate in biofilm formation, which are influenced by environmental factors, such as pH, temperature and concentration of nutrients in the medium. Based on the available literature and the observations from this study we hypothesize that other environmental and virulence factors are also crucial for biofilm production.

Standardization of Multiplex PCR:

The multiplex PCR has an advantage of detecting multiple genes in a single reaction (Townsend *et al.,* 2000). Further, it can be cost-effective and easy for handling. Hence, the multiplex PCR was optimized for detection of three genes *pil*B, *tad*B, *rcp*A with the same primers as listed in table 2. All the isolates and vaccine strain were positive for *pil*B, *tad*B and *rcp*A by amplifying product of size 113 bp, 158 bp and 191 bp respectively (Fig. 4).

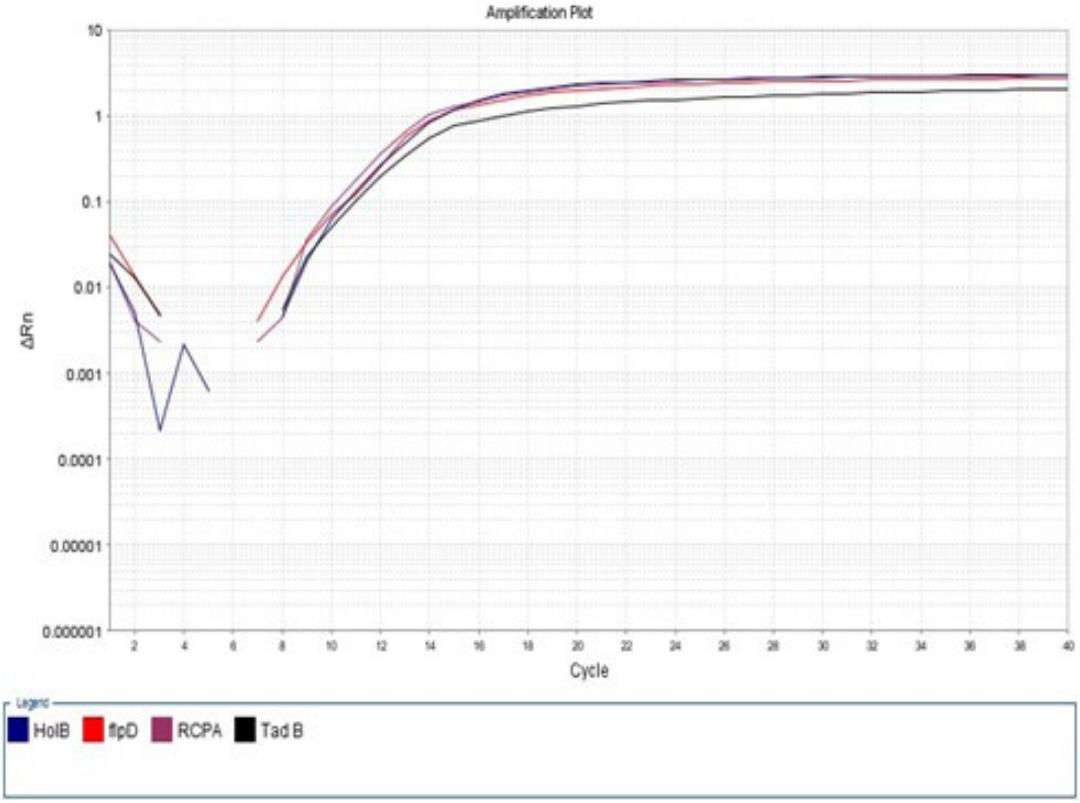

Fig. 1: The representative amplification plot for genes associated with biofilm formation.

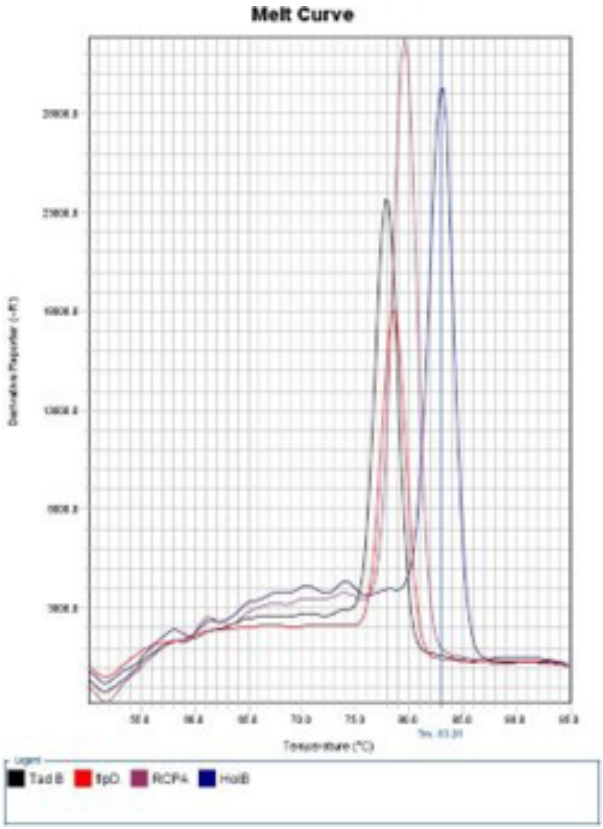

Fig. 2: The representative melt curve for genes associated with biofilm formation after real-time PCR.

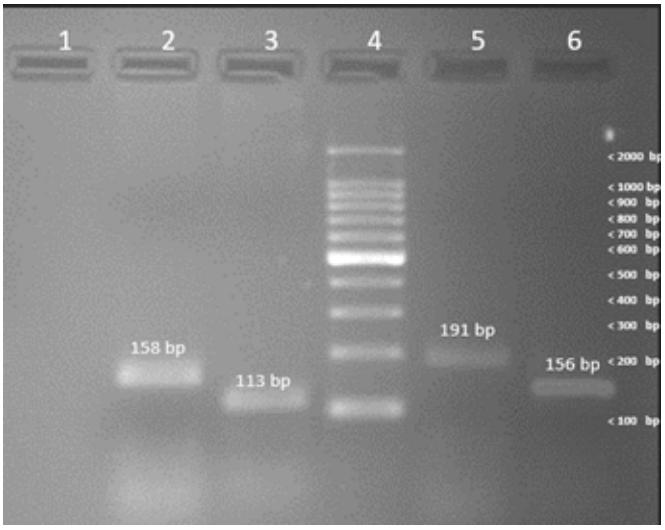

Fig. 3: The representative amplicon sizes for genes associated with biofilm formation on 2% agarose gel after real-time PCR. Lane 1 show no amplification for negative control, Lane 2 show amplicon size of 158 bp for tadB gene, Lane 3 show amplicon size of 113 bp for pilB gene, Lane 4 100 bp DNA ladder, Lane 5 show amplicon size of 191 bp for rcpA gene, Lane 6 show amplicon size of 156 bp for flpD gene.

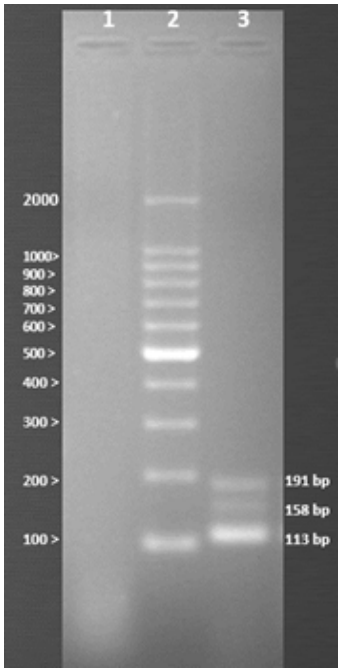

Fig. 4: The representative amplicon sizes for genes associated with biofilm formation on 2% agarose gel after multiplex PCR. Lane 1 show no amplification for negative control, Lane 2 is a100 bp DNA ladder, Lane 3 show amplicons of size 113 bp, 158 bp, 191 bp for *pil*B, *tad*B and *rcp*A respectively.

Conclusion

In conclusion, our study supports the consensus of the possible role of biofilm formation in evasion of treatment by *P. multocida* to develop chronic carrier status. This is the first real-time PCR for detection of genes associated with biofilm formation and demonstration of multiplex PCR for easy detection of three genes associated with biofilm formation in a single reaction in *P. multocida*. Our study indicates that there may be other factors involved that influence the formation of biofilm in addition to presence of biofilm genes in *P. multocida*.

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