STUDY ON PREVALENCE OF BIOFILM FORMING BACTERIA AND THEIR ANTIBIOTIC RESISTANCE PATTERN IN VETERINARY HOSPITAL ENVIRONMENT OF JAMMU REGION (INDIA)

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ABSTRACT

The objective of present study was to isolate and identify biofilm forming bacteria circulating in veterinary hospital environment and to determine their biofilm production potential. Thirty samples were collected from medical devices (n=10), table tops (n=5), animal trevis (n=5) and hand swabs of veterinary hospital workers (n=10) and analyzed for detection of numerous bacteria. Twenty four bacterial isolates comprising of both Gram positive and Gram negative bacteria were obtained. The prevalence of Gram positive bacteria was higher than Gram negative bacteria with the high occurrence of *Staphylococcus* spp. (43%) followed by *Escherichia coli* (23.3%), *Bacillus cereus* (6.7%), *Proteus* spp. (3.3%) and *Listeria monocytogenes* (3.3%). The biofilm production potential was assessed phenotypicaly by congo red agar method (CRA), tube method (TM) and microtitre plate method (MTP), the results varied in three tests and 41.6%, 70.8% and 91.6% samples showed biofilm production potential by CRA, TM and MTP, respectively. Antibiotic resistance pattern revealed 100% resistance to amoxicillin (penicillin group) whereas for cephalosporin group, resistance was shown *by B. cereus, Proteus*, *L. monocytogenes* and for cefoxitin, resistance was shown by *Staphylococcus*. *B. cereus*, *Staphylococcus* and *E. coli* were resistant to cefotoxaime. Therefore use of penicillin group drugs should be limited in the veterinary hospitals of Jammu region as bacterial isolates showed high resistance against them.

Key words: Hospital environment, biofilm, congo red agar, microtitre plate method

Introduction

The transmission of diseases from hospital environment to the fellow human beings, hospital staff, visitors and even outside environment of the hospital are well known. Many reports in human medicine have documented these findings and represent a great challenge for public health. Parallel to human medicine, the existence and transmission of infectious agents from veterinary hospitals environment is undeniable. Whereas, we are much aware of that hospital acquired infections are of great consequence in veterinary medicine due to several documented nosocomial outbreaks of various etiologies in both large and small animal veterinary hospitals (Goehring *et al*., 2010). However, the scientific reports on the infectious agents circulating in the veterinary hospitals' environment are limited. The presence and persistence of infectious agents in veterinary settings may compound the situation if the pathogens are of zoonotic in nature.

The persistence of bacterial pathogens in hospital environment, despite the intensive use of antiseptics and antibiotics, may involve the biofilm production. Biofilms are defined as complex communities of microorganisms (bacteria, fungi, algae, or protozoa) which attaches to biotic or abiotic surface by a matrix of exopolysaccharides secreted by these microorganisms (Dotsch *et al.,* 2012) and are one of the important pathogenicity factors associated with various bacteria. The formation of biofilms also protects the bacteria from immune system (Balaji *et al.,* 2013). Therefore biofilm forming bacteria in vertrinary hospitals may get transmitted to the hospital attendees and their animals. Also it has been suggested that bacteria can produce endotoxins once they are part of the biofilm (Gilbert *et al*., 2007). Biofilm production potential of pathogens has been explored to a great extent in human medicine and reported to be involved in 65 per cent of nosocomial infections (Hedayati *et al.,* 2014). The area is an emerging one in veterinary sciences. Therefore, it can be stated that there is need to explore much in the veterinary hospitals to for dectection of biofilm forming bacteria from the sites to which pet owners, pets and doctors come in contact more often. Hence present study was conducted with the objective to study the prevalance of various microorganisms in veterinary hospital vicinity of Jammu region and their biofilm forming potential which may cause various infections.

Materials and Methods

The study was carried out in the Division of Veterinary Public Health and Epidemiology, Faculty of Veterinary Sciences and Animal Husbandry, SKUAST-J, R.S. Pura, Jammu. A total of 30 samples were collected from Veterinary Hospital, R.S. Pura and Teaching Veterinary Clinical Complex, Faculty of Veterinary Sciences and Animal Husbandry, SKUAST-J. The samples were of animal trevis (n=5), hand swabs (n=10), table tops (n=5) and medical instruments (scissors, forceps, whelping forceps) (n=10). Samples were collected aseptically using sterilized swabs, the swabs were transported within two hours of collection to the Division of Veterinary Public Health and Epidemiology for further processing.

The bacterial isolates (n=24) obtained of various genera were subjected to detection of biofilm production potential by three phenotypic methods *viz.,* congo red agar method (CRA), tube method (TM) and microtitre plate method (MTP) as per protocols described by Freeman *et al.* (1989), Christensen *et al.* (1982) and O'Toole (2011), respectively with minor

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modifications. The black colonies with dry crystalline consistency were considered as biofilm positive for CRA whereas for TM, the sample was considered positive when a visible film lining was seen on the walls and bottom of tube. The positive results were categorized as weak, moderate and strong based on visual perception. Unlike congo red agar and tube method, microtiter plate method is a quantitative test, considered as a gold standard test among phenotypic methods for detection of biofilm.

The absorbance for MTP was measured at 492 nm using 30% acetic acid as control. Lastly, analysis of isolates, antibiogram pattern was performed by using disc diffusion technique (Bauer *et al.,* 1966). The antibiotics (n=10) of different groups - penicillin, fluoroquinolone, aminoglycosides and cephalosporin were used (HiMedia, Mumbai). The cultured isolates were spread plated on muller hinton agar (MHA) surface with sterile cotton swab and allowed to dry. Antibiotic discs were placed on MHA and incubated for 24 h at 37°C. The results were interpreted as per CLSI, 2014 and HiMedia antibiotic zone scale.

The results of microtiter plate method were interpreted as per following criteria:

Average OD value	Biofilm Production
ODs≤ ODc	No biofilm production
ODc< ODs ≤ 2 ODc	Weak biofilm production
2 ODc<ODs≤ 4 ODc	Moderate biofilm production
4 ODc≤ ODs	Strong biofilm production

ODs = Optical density of the sample
ODc = Optical density of the control

Results and Discussion

In the present study 30 samples from veterinary hospital environment/settings including medical devices (n=10), table tops (n=5), trevis (n=5) and hand swabs of the workers (n=10) were collected which revealed 24 bacterial isolates. Among twenty four bacterial isolates 16 were Gram positive bacteria and 8 were Gram negative bacteria, with high occurrence of *Staphylococcus* spp. (43%) followed by *E. coli* (23.3%), *B. cereus* (6.7%), *Proteus* spp. (3.3%) and *L. monocytogenes* (3.3%) (Table 1). These 24 bacterial isolates when subjected to congo red agar method for biofilm detection, revealed positive results in 10 isolates (41.7%) i.e. black colonies turning media black. Out of 13 *Staphylococcus* spp. only 8 showed biofilm production potential and the positive samples were from table top (n=2), trevis (n=2), hand swab (n=1) and medical instrument (n=3) followed by *E.coli* and *L. monocytogenes* on CRA (Table 2). However, with tube method 17 isolates were found to be positive for biofilm production (Table 3). Among 17 isolates, 16 revealed moderate production of biofilm and only one *Staphylococcus* isolate showed strong potential for biofilm production which was collected from trevis in comparison to the isolates collected from table and medical instruments. Further on subjecting these isolates to microtitre plate method (MTP) no isolate was found strong positive while one isolate (*E. coli* from trevis) was found to be moderately positive and rest 21(87.5%) isolates were found to be weak positive (Table 4).

These bacterial isolates were further subjected to various antibiotics commonly used in veterinary practice that fall under penicillin group (amoxicillin and amoxyclav), fluoroquinolone group (enrofloxacin and ciprofloxacin), aminoglycosides group (gentamicin and amikacin) and cephalosporin group (cefoxitin, cefotoxaime, ceftriaxone and ceftriaxone/ tazobactam). The study revealed that high percentage of bacterial isolates were resistant to amoxicillin (100%) followed by cefotaxime (Table 5). On Group wise analysis of antibiotics highest sensitivity of isolates was for aminoglycosides (sensitivity to gentamicin was higher than amikacin). Within cephalosporin group, sensitivity of isolates was for ceftriaxone and its combination with tazobactam; In fluoroquinolone group the sensitivity by the isolates towards ciprofloxacin and enrofloxacin was almost same except in the case of *Staphylococcus* spp. (more sensitive towards ciprofloxacin than enrofloxacin).

Bacterial pathogens found on analysis of 30 samples from veterinary hospitals settings belonged to various genera including *Staphylococcus* spp., *E. coli*, *L. monocytogenes*, *B. cereus*, *Proteus* spp. and many of them had the potential of forming biofilms. In our study higher occurrence of Gram positive bacteria than Gram negative bacteria was observed, with high occurrence of *Staphylococcus* spp. was record on investigation of 24 isolates obtained from 30 samples. *E. coli, B. cereus*, *Proteus* spp. and *L. monocytogenes* were also isolated. The findings were in aggrement with the findings of researchers from Pakistan, who reported high occurrence of *S. aureus* along with other *Staphylococcus* spp., *E. coli*, *Bacillus* spp., *Klebsiella pneumonia*, *P. aeruginosa,* and *Candida albicans* from the hospital environment in Karachi, Pakistan (Shaheen and Baqai 2016). Many other studies have reported that the hospital equipments and fomites may act as reservoir and source of nosocomial infectious agents and act as a reservoir such as stethoscopes (Fujita *et al*., 2013), thermometers (Van den Berg *et al*., 2000), multiple-dose vials (Sabino and Weese, 2006), white coats and surgical scrubs (Singh *et al*., 2013).

The present study investigated the presence of *Staphylococcus* spp. in hand swabs of workers, and 40% of the samples were positive for *Staphylococcus* spp. isolates this may be because of presence of *Staphylococcus* in nares of humans in contact with farm animals and the index finger has been implicated in transmission of *Staphylococcus* from nares to other humans via fomites or direct contact (Huber *et al*., 2010).

In our study, 41.7% isolates showed positive results in CRA, whereas 70.9 % (4.2% strong and 66.7% moderate) isolates were positive in TM. High rate of positivity was seen in TM than CRA which is also reported by Ruzicka *et al.* (2004) in MTP, 91.7% (4.2% moderate and 87.5% weak) isolates showed biofilm production potentital. MTP method detected highest number of biofilm positive isolates, this was in corroborate with the observations reported by the Goyal *et al*. (2014). MTP is considered as gold standard test for detecting biofilm production phenotypically (Mathur *et al.,* 2006). It is because the test is highly sensitive being based on the quantification of dye used in the test by spectrophotometer which may otherwise not be possible to measure by naked eyes (as done in TM).

Both Gram positive and Gram negative bacteria exhibited biofilm production potential. The study showed agreement with the findings of a researcher who reported the biofilm forming potentital among various Gram positive and Gram negative bacteria isolated from device associated infections as also

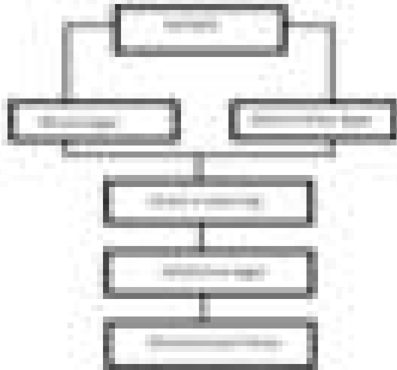

Fig. 1: Algorithm of isolation and identification of bacterial pathogens.

Table 1: Prevalence of bacteria isolated from different sources in veterinary hospital enviornment

Sample source (n)	Gram positive (n=16)		Gram negative (n=8)		
	Staphylococcus spp. (%)	*L. monocytogenes* (%)	*B. cereus* (%)	*E. coli* (%)	*Proteus* spp.(%)
Hand swabs(10)	4 (40.0)	0	0	0	0
Table tops (5)	3 (60.0)	0	1(20.0)	1(20.0)	1(20.0)
Trevis (5)	2 (40.0)	1 (20.0)	0	3(60.0)	0
Medical instruments (10)	4(40.0)	0	1 (10.0)	3(30.0)	0
30	13 (43.3)	1 (3.3)	2 (6.7)	7(23.3)	1(3.3)

Table 2: Biofilm production potential of isolates in congo red agar method

S. No.	Bacteria	No. of isolates	Congo red agar	
			Positive (%)	Negative (%)
1.	*Staphylococcus* spp.	13	8 (61.5)	5(38.5)
2.	*E. coli*	7	1(14.3)	35 (85.7)
3.	*B. cereus*	2	0 (0.0)	2(100)
4.	*Proteus* spp.	1	0 (0.0)	1(100)
5.	*L. monocytogenes*	1	1 (100)	0 (0.0)
	TOTAL	24	10 (41.7)	14 (58.3)

* Figures in parentheses indicate the per cent value

Table 3: Biofilm production potential of isolates in tube method

S. No.	Bacteria	No. of isolates	Tube Method		
			Strong	Moderate	Negative
1.	*Staphylococcus* spp.	13	1 (7.7)	8 (61.5)	4 (30.8)
2.	*E.coli*	7	0 (0.0)	6 (85.7)	1 (14.3)
3.	*B. cereus*	2	0 (0.0)	0 (0.0)	2 (100)
4.	*Proteus* spp.	1	0 (0.0)	1 (100)	0 (0.0)
5.	*L. monocytogenes*	1	0 (0.0)	1 (100)	0 (0.0)
	TOTAL	24	1 (4.2)	16 (66.7)	7 (29.1)

* Figures in parentheses indicate the per cent value

Table 4: Biofilm production potential of isolates in tube method

S. No.	Bacteria	No. of isolates	Microtiter plate method			
			Strong	Moderate	Weak	Negative
1.	*Staphylococcus* spp.	13	0 (0.0)	0 (0.0)	12(92.3)	1 (7.7)
2.	*E.coli*	7	0 (0.0)	1 (14.3)	6 (85.7)	0 (0.0)
3.	*B. cereus*	2	0 (0.0)	0 (0.0)	1 (50.0)	1(50.0)
4.	*Proteus* spp.	1	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)
5.	*L. monocytogenes*	1	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)
	TOTAL	24	0 (0.0)	1 (4.2)	21(87.5)	2 (8.3)

* Figures in parentheses indicate the per cent value

reported by Donlan (2001). Biofilm production was higher in *Staphylococcus* isolates than *E. coli* which was also reported by Shaheen and Baqai (2016) in hospital environment in Karachi.

The study revealed that high percentage of bacterial isolates were resistant to penicillin group (amoxicillin, 100%). This finding was in agreement with the findings of Windhal *et al.* (2015). Resistance to amoxicillin is observed in the present study which might be due to production of beta lactamase group of penicillin destroying enzymes. Thus, the present study

Table 5: Antibiotic resistance pattern of *Staphylococcus* spp., *E. coli, Proteus* spp., *L. monocytogenes* and *B. cereus* towards 10 antibiotics*

S. No.	Antibiotic Disc	Conc. (µg)	*Staphylococcus* spp. (n=13)			*E. coli* (n=7)			*Proteus* spp. (n=1)			*L. monocytogenes* (n=1)			*B. cereus* (n=2)		
			S	I	S	I	R	S	S	I	R	S	I	R	S	I	R
1	Amoxicillin	30	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	2 (100)
2	Amoxyclav	30	4 (30.8)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100)
3	Ciprofloxacin	5	6 (46.1)	4 (30.8)	1 (100)	0 (0.0)	0 (0.0)	1 (100)	1 (100)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)
4	Gentamicin	10	10 (76.9)	2 (15.4)	1 (100)	0 (0.0)	0 (0.0)	1 (100)	1 (100)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	2 (100)	0 (0.0)	0 (0.0)
5	Enrofloxacin	5	5 (38.5)	2 (15.4)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	1 (50.0)	0 (0.0)	1 (50.0)
6	Cefoxitin	30	6 (46.1)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	2 (100)
7	Ceftriaxone	30	9 (69.2)	4 (30.8)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100)	0 (0.0)
8	Cefotaxime	30	0 (0.0)	1 (7.7)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100)
9	Amikacin	30	7 (53.8)	3 (23.1)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	2 (100)	0 (27.3)	0 (0.0)
10	Ceftriaxone /Tazobactam	30/10	10 (76.9)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	1 (100)	1 (100)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	1 (50.0)	1 (50.0)

S= sensitive, I= intermediate, R= resistant; *Figures in parentheses represent percentage value

indicates the presence of bacterial pathogens in veterinary hospital settings, from where nosocomial transmission may occur and demands suitable preventive measures to be adopted to prevent such a transmission. Further, these bacterial pathogens circulating in veterinary hospital environment may have the potential of biofilm production and may require appropriate disinfection strategies to prevent it to happen.

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