Haryana Vet. (June, 2018) **57 (1)**, 76-79

Research Article

OCCURRENCE OF MULTIDRUG RESISTANT BIOFILM PRODUCING BACTERIA IN VETERINARY CLINICAL SETTINGS

ROHINI SHARMA, MANINDER SINGH*, S.K. KOTWAL, M.A. MALIK and HEENA CHAMBYALIA
Division of Veterinary Public Health and Epidemiology
Sher-e-Kashmir University of Agricultural Sciences and Technology, Jammu-181102, India

ABSTRACT

The present study assessed the occurrence of multidrug resistant bacteria in veterinary clinical settings along with their biofilm production potential. A total of 118 bacterial isolates. The recovered isolates belonged to 8 bacterial genera/species *viz., Staphylococcus* spp. (n=43), *Streptococcus* spp. (n=4), *Bacillus cereus* (n=11), *Escherichia coli* (n=41), *Listeria monocytogenes* (n=1), *Pseudomonas aeruginosa* (n=2), *Klebsiella* spp. (n=6) and *Proteus* spp. (n=10). In veterinary clinical cases, Gram negative bacteria predominated whereas in veterinary hospital environment Gram positive bacteria predominated. On analysis of 118 bacterial isolates for biofilm production potential by microtiter plate method, 94.1% (n=111) isolates showed weak positive results. Antibiotic resistance pattern of 111 isolates revealed 100% resistance to amoxicillin followed by cefotaxime (76.95%), cefoxitin (65.87%) and amoxyclav (55.25%).

Key words: Biofilm, microtiter plate method, antibiotic resistance, clinical cases, hospitals

The persistence of bacterial pathogens in clinical cases and hospital environment, despite the extensive use of antiseptics and antibiotics, may involve the biofilm formation. A biofilm is an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with surface and enclosed in a matrix of polysaccharide. Biofilms may form on a wide variety of surfaces including living tissues and indwelling medical devices (Donlan, 2001). The biofilms are reported to have high resistance to antimicrobial agents and less sensitivity to inhibitors (Jabra-Rizkn *et al.,* 2006).

The pathogens found in veterinary hospitals and clinical cases are numerous and include *Staphylococcus* spp. which may or may not be methicillin resistant, *Escherichia coli, Enterococcus* spp., *Pseudomonas aeruginosa*, *Streptococcus* spp. etc. Padhy *et al.,* 2014; Shaheen and Baqai, 2016). These pathogens are reported to cause various infections in animals such as mastitis urinary tract infections (Nam *et al.,* 2013), delayed wound healing (Padhy *et al.,* 2014), surgical site infections (Smith and Ross, 2002), diarrhea (Weese and Armstrong, 2003) etc., and many of them have potential of forming biofilms (Mathur *et al.,* 2006; Oliveria *et al.,* 2014). In a study by Shaheen and Baqai (2016), the persistence of *Staphylococcus aureus* and *Candida albicans* in hospitals is very much related to biofilm formation. Thus, the drug resistant and biofilm forming bacteria have been reported in veterinary hospitals and its environment across the world from where these pathogens could easily be transmitted and disseminated to animal and human attendants KuKanich *et al.,* 2012; Oliveria *et al.,* 2014). Therefore, the present study was conducted to study the occurrence of biofilm producing bacteria along with their antibiotic resistance pattern in veterinary clinical settings in and around Jammu.

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 170 samples from veterinary clinical cases {diarrhea (n=45), mastitis (n=45), wounds (n=30), otitis externa (n=17) and cystitis (n=3)} and veterinary hospital environment {table tops (n=5), trevis (n=5), medical instruments (n=10) and hand swabs (n= 10)} were collected aseptically using sterilized swabs. The swabs were transported to the laboratory under chilled conditions within two hours of their collection for further processing. The swabs were streaked on blood agar and MacConkey agar and incubated for 24-48 hrs at 37°C. Preliminary identification of bacteria was done on the basis of colony morphology, size and characteristic growth on blood agar and MacConkey agar followed by Gram staining. The presumed colonies were streaked on selective agar (Mannitol salt agar for *Staphylococcus* spp. Polymyxin pyruvate egg yolk mannitol bromothymol blue agar for *Bacillus cereus* MacConkey agar for *Klebsiella* spp. Eosin metylene blue agar for *E. coli* and Listeria oxford medium base modified agar for *Listeria monocytogenes* and incubated at 37°C for 24-48 hrs followed by Gram staining and biochemical characterization (catalase test, oxidase test and IMViC test. The confirmed bacterial isolates (n=118) of various genera were subjected to detection of biofilm production potential by microtiter plate method (MTP) (O'toole, 2011) as this method is considered as gold standard test

The results of MTP 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

*Corresponding author: manindersingh2k2@gmail.com

Table 1
Biofilm production potential of bacteria isolated from veterinary clinical cases and hospital environment (n=118)*

S.No.	Bacteria	No. of isolates	Microtiter plate method			
			Strong	Moderate	Weak	Negative
1.	*Staphylococcus* spp.	43	0	1 (2.3)	40 (93.0)	2 (4.7)
2.	*Streptococcus* spp.	4	0	0	3 (75.0)	1 (25.0)
3.	*E.coli*	41	0	4 (9.8)	37 (90.2)	0
4.	*B. cereus*	11	0	0	8 (72.7)	3 (27.3)
5.	*P. aeruginosa*	2	0	0	2 (100)	0
6.	*Klebsiella* spp.	6	0	0	5 (83.3)	1 (16.7)
7.	*Proteus* spp.	10	0	1 (10.0)	9 (90.0)	0
8.	*L. monocytogenes*	1	0	0	1 (100)	0
	TOTAL	118	0	6 (5.1)	105(89.0)	7 (5.9)

* Figures in parentheses indicate the percentage

for detecting biofilm production phenotypically (Mathur *et al*., 2006). The results of MTP were interpreted as per following criteria:-

The absorbance for MTP was measured at 492 nm using 30% acetic acid as control.

The isolates having biofilm production potential were analyzed for antibiogram pattern using 10 antibiotic discs (HiMedia, Mumbai) belonging to penicillin group (amoxicillin and amoxyclav), fluoroquinolone group (enrofloxacin and ciprofloxacin), aminoglycosides group (gentamicin and amikacin) and cephalosporin group (cefoxitin, cefotoxaime, ceftriaxone and ceftriaxone/ tazobactam) by disc diffusion technique (Bauer *et al.,* 1966). The zone diameter of each antibiotic was interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, 2014).

RESULTS AND DISCUSSION

A total of 118 bacterial isolates (94 from veterinary clinical cases and 24 from hospital environment) were recovered from 170 samples originated from veterinary clinical cases {diarrhea (n=45), mastitis (n=45), wounds (n=30), otitis externa (n=17) and cystitis (n=3)} and veterinary hospital environment {table tops (n=5), trevis (n=5), medical instruments (n=10) and hand swabs (n= 10)}, The confirmed bacterial isolates (n=118) belonged to various genera *viz.*, *Staphylococcus* spp. (n=43), *Streptococcus* spp. (n=4), *E. coli* (n=41), *L. monocytogenes* (n=1), *Pseudomonas* spp. (n=2), *Klebsiella* spp. (n=6), *B. cereus* (n=11) and *Proteus* spp. (n=10). These findings corroborate with the findings of Nam *et al.* (2013), Padhy *et al.* (2014), Smith and Ross (2002), Weese and

Table 2
Antibiotic resistance pattern of biofilm producing bacterial isolates (n=111) towards selected antibiotics*

Antibiotic Disc	Conc. (µg)	*Staphylococcus* spp. (n=41)	*Streptococcus* spp. (n=3)	*E. coli* (n=41)	*P. aeruginosa* (n=2)	*Klebsiella* spp. (n=5)	*Proteus* spp. (n=10)	*L. monocytogenes* (n=1)	*B. cereus* (n=8)
Amoxicillin	30	41 (100)	3(100)	41 (100)	2 (100)	5 (100)	10 (100)	1 (100)	8 (100)
Amoxyclav	30	30 (73.2)	0	20 (48.8)	2 (100)	4 (80.0)	4 (40.0)	0	8(100)
Ciprofloxacin	5	12 (29.3)	0	8 (19.5)	0	1 (20.0)	0	0	0 (0.0)
Gentamicin	10	3 (7.3)	0	0	0	2 (40.0)	0	0	0 (0.0)
Enrofloxacin	5	21 (51.2)	0	9 (22.0)	0	1 (20.0)	2 (20.0)	0	3 (37.5)
Cefoxitin	30	25 (61.0)	2(66.7)	12 (29.3)	2 (100)	2 (40.0)	3 (30.0)	1 (100)	8 (100)
Ceftriaxone	30	0	3(100)	3 (7.3)	0	4 (80.0)	4 (40.0)	0	5 (62.5)
Cefotaxime	30	40 (97.6)	3 (100)	3 (7.3)	2 (100)	5 (100)	4(40.0)	0	8 (100)
Amikacin	30	10 (24.4)	1(33.3)	0	0	0	0	0	0 (0.0)
Ceftriaxone /Tazobactam	30/10	9 (22.0)	0	19 (46.3)	0	2 (40.0)	0	0	6 (75.0)

* Figures in parentheses indicate the percentage

Armstrong (2003), Kumar *et al*. (2010) and Shaheen and Baqai (2016), who reported the isolation of these bacteria from mastitis, cystitis, wounds, surgical site infections, diarrhea, otitis and hospital environment, respectively. High occurrence of *Staphylococcus* spp. have also been reported by Dar *et al*. (2014) and Shaheen and Baqai (2016) from clinical cases of mastitis and hospital environment, respectively.

Out of 118 isolates, 111 were found to be positive for biofilm formation by MTP method. A greater proportion of isolates *i.e.* 89% (n=105), were weak biofilm producers whereas only 5.1% (n=6) of isolates showed moderate biofilm production potential. None of the isolates showed strong biofilm production potential by MTP method (Table1). MTP is considered as a very sensitive test for detection of biofilm production potential and is based on quantification of dye used in the test by spectrophotometer (Hassan *et al*., 2011). These results of biofilm detection by MTP method corroborate with the observations of Oliveira *et al.* (2010) and Goyal *et al*. (2014). Both Gram negative and Gram positive bacteria showed biofilm formation potential and this finding is in agreement with observations of Donlan (2001) and Hassan *et al*. (2011) who reported biofilm formation potential among various Gram positive and Gram negative bacteria (*Staphylococcus*, *E. coli, P. aeruginosa, K. pneumonia* and *E. fecalis)* isolated from device associated infections and clinical samples. As observed in the present study, Shaheen and Baqai (2016) and Hassan *et al*. (2011) also reported high percentage of *Staphylococcus* isolates positive for biofilm formation as compared to *E. coli* isolates (Table 1).

On further subjecting biofilm producing bacterial isolates (n=111) to antibiotic sensitivity testing, it was found that high percentage of bacterial isolates were resistant to amoxicillin (100%) followed by cefotaxime (76.95%), cefoxitin (65.87%) and amoxyclav (55.25%). The findings of the present are in agreement with the observations of Hussain *et al*. (2015) and Milton *et al*. (2015), who also reported resistance of *Staphylococcus* spp., *Klebsiella* spp., *P. aeroginosa*, *Proteus* spp. and *E. coli* to amoxicillin, cefotaxime, amoxyclav and cefoxitin. The resistance observed to amoxicillin in the present study might be due to production of beta-lactamase group of penicillin destroying enzymes.

The group-wise analysis of antibiogram pattern revealed low degree of resistance of bacterial isolates to aminoglycosides (gentamicin and amikacin). Within cephalosporin group, resistance of isolates was highest for cefotaxime (76.95%) followed by cefoxitin (65.87%) and ceftriaxone (36.22%). In a study conducted by Hussain *et al*. (2015) in Karachi, Pakistan, the bacteria isolated from various clinical cases (ear and eye infection swabs, wound swabs, fecal samples and pus samples) were also found to be resistant to third generation cephalosporins. *E. coli* was found to be resistant to cefotaxime (67%) and ceftriaxone (67%); *Proteus* spp. to cefotaxime (60%) and ceftriaxone (60%) and *S. aureus* to ceftriaxone (77%) and cefotaxime (50%). In the present study, *E. coli*, *Proteus* and *S. aureus* exhibited high resistance to cefotaxime (78%, 40% and 97.6%, respectively) while the resistance was comparatively much lower against ceftriaxone (7.3%, 40% and 0%, respectively) (Table 2).

The resistance to antibiotics by bacteria is their inherent ability which depends on numerous factors. Resistance can appear spontaneously due to random mutations and irrational use of antibiotics. Nonprudent use of drugs in communities, animal husbandry and fishery practices may result in emergence and spread of antibiotic resistant organisms that may lead to therapy failure and increase in morbidity rates (Munita and Arias, 2016). Apart from these, the formation of biofilms in bacteria may aid to protect them from environmental stresses like antibiotics and immune system (Balaji *et al.,* 2013). It is reported that extracellular polymeric substances (EPS) in biofilms provides a barrier to penetration of antibiotics either by reacting chemically with the antimicrobial molecules or by limiting their rate of transport resulting in inefficacy of antimicrobials (Donlan, 2001).

Thus, the present study revealed the presence of multidrug resistant bacteria in veterinary clinical cases and hospital settings and their biofilm formation potential which may result in nosocomial transmission of these pathogens. Therefore, appropriate disinfection strategies and necessary preventive measures need to be adopted to prevent such transmissions. Also, it is important to select antibiotics on the basis of susceptibility tests so as to prevent the emergence of multidrug resistant bacteria in clinical cases.

ACKNOWLEDGMENT

We are thankful to Dean, F.V.Sc & A.H., SKUAST-J for financial assistance and infrastructure facilities to execute this study.

REFERENCES

Balaji, K., Thenmozhi, R. and Pandian, S. K. (2013). Effect of subinhibitory concebtrations of fluoroquinolones on biofilm production by clinical isolates of *Streptococcus pyogens. Indian J. Med. Res.***137**: 963-971.

Bauer, A.W., Kirby, W. M. M, Sheris, J. and Truck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.***145**: 225-230.

CLSI (Clinical and Laboratory Standards Institute). (2014). Performance Standards for Antimicrobial Susceptibility Testing, 24th informational supplement, M100-S24. Wayne, PA: Clinical and Laboratory standards Institute.

Dar, K. H., Ansari, M. M., Dar, S. H., Tantary, H. A., Baba, M. A. and Naikoo, M. U. D. 2014. Studies on subclinical mastitis in dairy cows of Jammu and Kashmir. *Int. J. Vet. Sci.* **3(2)**: 95-99.

Donlan, R. M. (2001). Biofilms and Device-Associated Infections. *Emerg. Infect. Dis.***7(2)**: 277-281.

Fabres-Klein, M. H., Santos, M. J. C., Raphael, C. K., Guilherme, N. D. S. and Ribon, A. B. R. (2015). An association between milk and slime increases biofilm production by bovine *Staphylococcus aureus*. *BMC Vet. Res.***11**: 3. doi: 10.1186/s12917-015-0319-7.

Goyal, R., Kerketta, P., Kumar, P., Rawat, M., Viswas, N. K. and Agarwal, R. K. (2014). Genotypic and phenotypic characterization of clinical isolates of *Staphylococcus aureus* for biofilm formation ability. *Adv. Anim. Vet. Sci.* **2(4)**: 233-238.

Hassan, A., Usman, J., Kaleem, F., Omair, M., Khalid, A. and Iqbal, M. (2011). Evaluation of different detection methods of biofilm formation in the clinical isolates. *Braz. J. Infect. Dis.* doi: 10.1016/S1413-8670(11)70197-0.

Hussain, A., Razvi, N., Anjum, F. and Humayoun R. (2015). Resistance pattern of 3rd generation cephalosporins. *World J. Pharm. Pharm. Sci.* **4(4)**: 34-44.

Jabra-Rizk, M. A., Meiller, T, F., James, C. E. and Shirtliff, M. E. (2006). Effect of farnesol on *Staphylococcus aureus* biofilm formation and antimicrobial susceptibility. *Antimicrob. Agents Chemother.* **50**:1463-1469.

KuKanich, S. K., Ghosh, A., Skarbek, J. V., Lothamer, K. M. and Ludek, Z. (2012). Surveillance of bacterial contamination in small animal veterinary hospitals with special focus on antimicrobial resistance and virulence traits of enterococci. *J. Am. Vet. Med. Assoc.* **240**: 437-445.

Kumar, K. S., Selvaraj, P., Vairamuthu, S., Shammi, M. and Kathiresan, D. (2010). Antibiogram patterns of microbes isolated from otitis externa of dogs. *Indian J. Vet.Ani. Sci. Res.,* **6(3)**: 145-147.

Mathur, T., Singhal, S., Khan, S., Upadhyay, D. J., Fatma, T. and Rattan, A. (2006). Detection of biofilm formation among the clinical isolates of Staphylococci: An evaluation of three different screening methods. *Indian J. Med. Microbiol.* **24(1):** 25-29.

Milton, A. A. P., Priya, G. B., Aravind, M., Parthasarathy, S., Saminathan, M., Jeeva, K. and Agarwal, R. K. (2015). Nosocomial infections and their surveillance in veterinary hospitals. *Adv. Anim. Vet. Sci.* **3(2)**: 1-24.

Munita, J. M. and Arias, C. A. (2016). Mechanisms of Antibiotic Resistance. *Microbiol.Spectr*. 4(2): 1-37

Nam, E.H., Ko, S., Chae, J.S. and Hwang, C.Y. (2013). Characterization and zoonotic potential of uropathogenic *Escherichia coli* isolated from dogs. *J. Microbiol. Biotechnol.* **23**: 422-429.

Oliveria, A. and Cunha, M. de L.R. (2010). Comparison of methods for the detection of biofilm production in coagulase – negative staphylococci. *BMC Res. Notes.* **3:** 260.doi: 10.1186/1756-0500-3-260.

O'Toole, G. A. (2011). Microtiter Dish Biofilm Formation Assay. *J. Vis. Exp.*doi: 10.3791/2437.

Padhy, A., Mishra, R., Behera, S. S., Sahu, A. R. and Sahoo, S. (2014). Microbial profile of canine persistent wound infections. *Vet.World*. **7(4)**:244-247.

Smith, M. A. and Ross, M. W. (2002). Postoperative infection with *Actinobacillus* spp. in horses: 10 cases (1995-2000). *J. Am. Vet. Med. Assoc* .**221(9)**: 1306-1310.

Shaheen, A. and Baqai, R. (2016). BiofilmfFormation by environmental microbes isolated from hospitals in Karachi, Pakistan. *Am. Sci. Res. J. Eng. Technol. Sci.* **15(1)**: 240-251.

Weese, J. S. and Armstrong J. (2003). Outbreak of *Clostridium difficile*–associated disease in a small animal veterinary teaching hospital. *J. Vet. Intern. Med.***17**: 813-816.