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บทความวิจัย

การตรวจวิเคราะห์เชื้อไบโอฟิล์มจากฟันสุนัขด้วยวิธี Congo red tube method และ tissue culture plate method

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คำสำคัญ
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บทคัดย่อ

เชื้อไบโอฟิล์มเป็นกลุ่มแบคทีเรียที่เกาะติดกับฟันสุนัขซึ่งเป็นสาเหตุทำให้เกิดการอักเสบของเหงือกและโรคเหงือกอักเสบในสุนัข การสัมผัสน้ำลายสุนัขอาจทำให้เกิดการติดต่อเชื้อไบโอฟิล์มทั้งระหว่างสุนัขและเจ้าของหรือติดต่อระหว่างสุนัขด้วยกันเอง การศึกษานี้มีวัตถุประสงค์เพื่อตรวจวิเคราะห์เชื้อไบโอฟิล์มและประเมินความเหมาะสมของวิธีที่ใช้ตรวจวิเคราะห์จากทั้งสามวิธีได้แก่ วิธี Congo red agar (CRA) Tube method (TM) และ Tissue culture plate (TCP) โดยการศึกษานี้ วิธี TCP ถูกใช้เป็นวิธีมาตรฐาน (gold method) ในการเปรียบเทียบผลของตรวจวิเคราะห์เชื้อไบโอฟิล์ม จากผลการศึกษาพบว่า วิธี TCP ตรวจพบเชื้อไบโอฟิล์ม จำนวน 46 ตัวอย่าง คิดเป็น 38.98 % การตรวจด้วยวิธี CRA พบเชื้อไบโอฟิล์ม จำนวน 19 ตัวอย่าง คิดเป็น 16.10 % และไม่พบเชื้อไบโอฟิล์ม จำนวน 99 ตัวอย่าง คิดเป็น 83.90 % เมื่อเทียบกับวิธี TCP วิธี CRA มีความไวและความจำเพาะเจาะจง เท่ากับ 41% และ 100% ตามลำดับ ส่วนวิธี TM ตรวจพบเชื้อไบโอฟิล์ม จำนวน 32 ตัวอย่าง คิดเป็น 27.12 % และไม่พบเชื้อไบโอฟิล์ม จำนวน 86 ตัวอย่าง คิดเป็น 72.88 % เมื่อเปรียบเทียบกับวิธี TCP วิธี TM มีความไวและความจำเพาะเจาะจง เท่ากับ 69 % และ 100 % ตามลำดับ จากผลการศึกษาพบว่าเมื่อเปรียบเทียบระหว่างวิธี TM และ CRA พบว่า วิธี TM มีประสิทธิภาพมากกว่าวิธี CRA ซึ่งเมื่อเปรียบเทียบทั้ง 3 วิธี พบว่าวิธี TCP มีประสิทธิภาพมีความจำเพาะ และความไวมากที่สุด นอกจากนี้ยังน่าเชื่อถือและง่ายในการตรวจวิเคราะห์เชื้อไบโอฟิล์มจากฟันสุนัขซึ่งสามารถนำไปใช้ในการตรวจวิเคราะห์เชื้อไบโอฟิล์มเบื้องต้นในห้องปฏิบัติการทางสุขภาพสัตว์ต่อไป

Introduction

A biofilm is a group of bacteria that adheres to the canine tooth surface, known as plaque, and can lead to canine gingivitis and periodontal disease. The biofilm produces extracellular polymeric substances (EPS), creating a sticky mucus and highly complex structures that protect against antibiotic diffusion within the bacterial group (Papenfort & Bassler, 2016). The bacteria within the biofilm communicate with each other through a quorum-sensing system (Watson et al., 2002). The biofilm structure exhibits strong antibiotic resistance, which hinders the penetration of antibiotics into the biofilm layer. Biofilms can lead to recurrent infections and chronic contagious diseases, making treatment challenging. Bacterial biofilms have had detrimental effects on both the animal and economic industries, causing issues such as clinical mastitis in cows, canine dermatitis, canine gingivitis, and periodontal disease.

In particular, contact with pets, involving touching and nasal ingestion, can lead to diseases (Goldstein et al., 1978; Meyers et al., 2008). The canine's oral cavity often accumulates plaque on the teeth and gums, forming a community of various bacterial strains that play a crucial role in infectious diseases between animals and humans (Offenbacher et al., 1996). Bacterial biofilm has been isolated in canines, including *P. aeruginosa* (Charlotte et al., 2013), *S. intermedius*, *S. epidermis*, *S. canis* (Elizabeth et al., 2014),

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S. mutans (Bai et al., 2016), and *S. pseudointermedius* (Osland et al., 2012; Han et al., 2015). A specific method for isolating biofilm from dog teeth allows for the rapid identification of infections and disease prevention in pet owners.

There are several basic biofilm identification methods, including bacterial culture on Congo Red Agar (CRA), in vitro culture in the test tube (Tube Method TM) (Mohamed et al., 2016), the 96 well tissue culture plate (TCP) (Triveda and Gomathi, 2016). TCP has been used as the gold standard for biofilm detection (Dhanalakshmi et al., 2018). However, the majority of biofilm identification methods have been developed and studied in humans, with limited application in canine. Therefore, this study aims to detect biofilm-producing bacteria in canine teeth and to evaluate three methods CRA, TM, and TCP for their suitability in a screening test for biofilm isolation in veterinary diagnostic laboratories.

Materials and methods

Bacterial preparation

In this study, bacteria samples were collected from canine teeth of dogs without limitation on age, gender, and species by cotton swab method at talat sub-district, mueng district, and Maha Sarakham province, Thailand. On blood agar (BA) (Himedia, India), the swabs are incubated and maintained at 37°C for 18-24 hours. The single colonies were cultured on trypticase soy agar (TSA) (Difco, USA) and then incubated at 37 °C for 18-24 hours. One hundred eighteen isolates were separated from this study. *S. intermedius* (TISTR 668) was used as positive control. The isolates were stored in 20 % glycerol at -20 °C, pending further CRA, TM, and TCP screening of biofilm-producing bacteria.

Biofilm production analysis

Biofilm production by isolated canine teeth was detected using three phenotypic methods, including TCP, TM, and CRA. Biofilm production was categorized into four grades: strong, moderate, weak, and non-biofilm. Strong and moderate results were interpreted as indicative of biofilm production, while weak and non-biofilm production were categorized as non-biofilm.

Congo Red Agar (CRA) method

The preparation of the CRA medium was adjusted according to Freeman et al. (1989) who included 37 g/L of brain heart infusion (BHI) agar (Himedia, India), 30 g/L of Sucrose (Ajax, Australia), 12.5 g per liter of agar no.1 (Himedia, India), and 8 grams per liter of congo red indicator (Sigma, India). The Congo red indicator solution was prepared separately and sterilized at 121°C for 15 minutes, then added to the BHI and sucrose solution at 55°C. A total of 118 purified colonies were streaked on the CRA-specific biofilm media and maintained at 37°C for 24-48 hours. The colonies appearing black on the CRA medium were indicative of biofilm-producing bacteria, sometimes accompanied by the presence of sticky mucus. Non-biofilm bacteria were characterized by red colonies (Freeman et al., 1989). The CRA culture was conducted three times in triplicate.

Tube Method (TM)

The biofilm screening technique in this assay was adapted from Christensen et al. (1985). An entire loop of each of the 118 isolates was transferred to 10 mL of trypticase soy broth (TSB) (Himedia, India) mixed with 1 % glucose (Univar, Australia), and then incubated at 37°C for 24-48 hours. The incubated tubes were poured, and the suspension was gently washed with phosphate-buffered saline (pH 7.2) three times. The tested tubes were stained with 0.10 % crystal violet for 30 minutes and rinsed to remove excess color with deionized water, followed by air-drying. The intensity characteristics of the crystal violet coating on the test tube's rim were recorded. The scoring was as follows: a score of 0 indicated no crystal violet coloration, defining non-biofilm-producing bacteria (none). A score of 1 indicated light attachment, signifying low biofilm-producing bacteria (weak). A score of 2 indicated medium-dark coloration, defining moderate biofilm-producing bacteria, and a score of 3 indicated dark-colored crystal violet, signifying potent biofilm-producing bacteria (Mohamed et al., 2016). The procedure was repeated three times in triplicate.

Tissue Culture Plate Method (TCP)

The biofilm screening technique in the TCP method was adapted from Christensen et al. (1985). All 118 isolates were cultured in trypticase soy broth (TSB) (Himedia, India) mixed with 1 % glucose (Univar, Australia). The bacterial concentration was adjusted to match that of McFarland standard no. 2. Two hundred microliters of isolated bacteria were transferred into each well of a 96-well tissue culture plate. In the negative control group, the wells were left unfilled. The plates were incubated at 37°C for two days. After incubation, the bacterial suspension was rinsed and washed thrice in phosphate-buffered saline (PBS) with a pH of 7.2. The plate was then stained with 0.10 % crystal violet, rinsed to remove excess dye, and washed with deionized water before being left to air dry. Next, 200 microliters of 1 % citric acid were added to each well and gently mixed. The 96-well plates were used to measure the absorbance with a microplate reader (UVM340, microplate reader, ASYS, Biocompare, USA) at a wavelength of 620 nm. Biofilm formation was classified into four categories following Stepanovic et al. (2000): non-biofilm (OD < ODc), weak biofilm (ODc < OD < 2x ODc), moderate biofilm (2x ODc < OD < 4x ODc), and strong biofilm (OD > 4x ODc). The value ODc was calculated and analyzed using ODc = average OD of the negative control + 3 standard deviations of the negative control (Mohamed et al., 2016). The procedure was carried out three times in triplicate. Grading of biofilm formation by tissue culture plate method was shown in Table 1.

Table 1 Grading of biofilm formation by tissue culture plate method

Optical densities values	Adherences	Biofilm formation
< 0.120	Non	Non/weak
0.120-0.240	Moderate	Moderate
> 0.240	Strong	Strong

Statistical analysis

In the present study, TCP was considered the gold standard method for biofilm detection and was compared with data from TM and CRA. Accordingly, the data from TCP were compared with that from TM and CRA. The data were presented as numbers and %. Parameters such as sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for each test using a conventional two-by-two (2X2) table (Parikh et al., 2008). True positives were identified as biofilm production detected by the TCP, TM, and CRA methods. False positives were instances of biofilm production detected by the TM and CRA methods but not by the TCP method. False negatives were isolates that did not produce biofilm according to the TM and CRA methods but did when assessed with the TCP method. True negatives were cases where no biofilm was produced according to all three methods. Apparent and true prevalence were analyzed according to Habibzadeh et al. (2022).

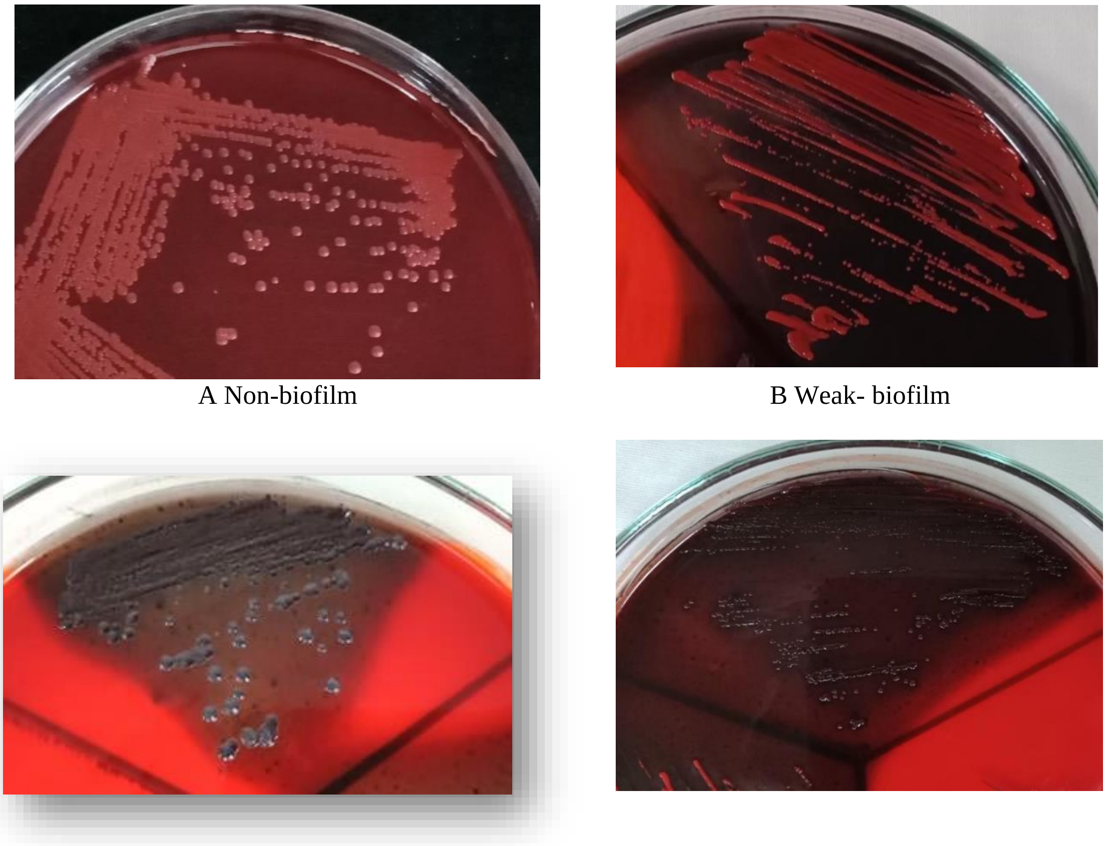
Results and Discussion

Gingivitis disease, periodontal diseases, and dental caries are oral conditions caused by microorganisms that can affect every dog at some point during its lifetime. The primary approach for treating and preventing these diseases is the mechanical removal of bacteria.

Antimicrobial treatment alone may not be effective for many other bacterially influenced disorders due to two main reasons: First, oral bacterial diseases involve multiple types of bacteria (no single microorganism is the sole cause), and second, reinfection is inevitable because the pathogens are part of the natural oral microflora (Lamont et al., 2019; Lasserre et al., 2018).

This study involved the detection of the biofilm-forming abilities of bacterial isolates from the canine teeth of 39 dogs, resulting in the purification and identification of 118 bacterial strains. Furthermore, we compared the efficiency of the TCP-based biofilm screening method with other methods such as CRA and TM. Congo Red Agar (CRA) medium was used to culture 118 isolates, which were then incubated for 24-48 hours at 37°C. The phenotypic colony characteristics of biofilm on CRA were presented as Figure 1A-D. Among the biofilm-producing bacteria, 19 out of the 118 isolates (16.10 %) were categorized as strong, 62 out of 118 isolates (52.54 %) as weak, and 37 out of 118 isolates (31.35 %) as non-biofilm producers (Table 2). Out of the 118 isolates tested with CRA culture media, 19 isolates (16.10 %) were classified as biofilm-production, while 99 isolates (83.90 %) were identified as non-biofilm-producing bacteria (Table 3). Using the TM method, it was determined that 2 out of 118 isolates (1.69 %) exhibited high-level biofilm production. Medium-level biofilm production characteristics were observed in 30 isolates, accounting for 25.42 %, and were categorized as moderate biofilm producers. A total of 50 isolates (42.37 %) displayed faint color, indicating weak biofilm formation, while 36 isolates (30.50 %) showed no biofilm formation, and they were classified as non-biofilm producers (Table 2). Out of the 118 isolates tested with TM, 32 isolates (27.12 %) were classified as biofilm-production, while 86 isolates (72.88 %) were identified as non-biofilm-producing bacteria (Table 3). The coloring phenotypic of biofilm on TM method are illustrated in Figure 2. The bacterial culture was introduced into a 96-well tissue culture plate (TCP), and positive biofilm formation was observed around the well. Adherent bacteria were stained with crystal violet and examined using a microplate reader at 620 nm. The results indicated the impact of strong, moderate, weak, and non-biofilm formation, with 21 out of 118 isolates (17.79 %), 25 out of 118 isolates (21.19 %), 30 out of 118 isolates (25.42 %), and 42 out of 118 isolates (35.59 %), respectively (Table 2). Out of the 118 isolates tested with TCP, 46 isolates (38.98 %) were classified as biofilm-production, while 72 isolates (61.02 %) were identified as non-biofilm-producing bacteria (Table 3). The color grading phenotypic of biofilm on the TCP method is depicted in Figure 3.

When compared with TCP, CRA truly detected 19 biofilm producers and 72 non-biofilm producers, while TM truly identified 32 biofilm producers and 72 non-biofilm producers (Table 3). In this study, TCP was used as the gold standard for biofilm detection (Dhanalakshmi et al., 2018). The performance characteristics of CRA and TM when compared with TCP are presented in Table 4 and 5. CRA exhibited a sensitivity of 41 %, specificity of 100 %, PPV of 100 %, NPV of 72 %, AP of 0.16 and TP of 0.39, while TM showed a sensitivity of 69 %, specificity of 100 %, PPV of 100 %, NPV of 83 %, AP of 0.12 and TP of 0.39 (Table 6).



A Non-biofilm B Weak- biofilm

C Strongly-biofilm D Positive control

Figure 1 Colony Characteristics of biofilm on CRA.

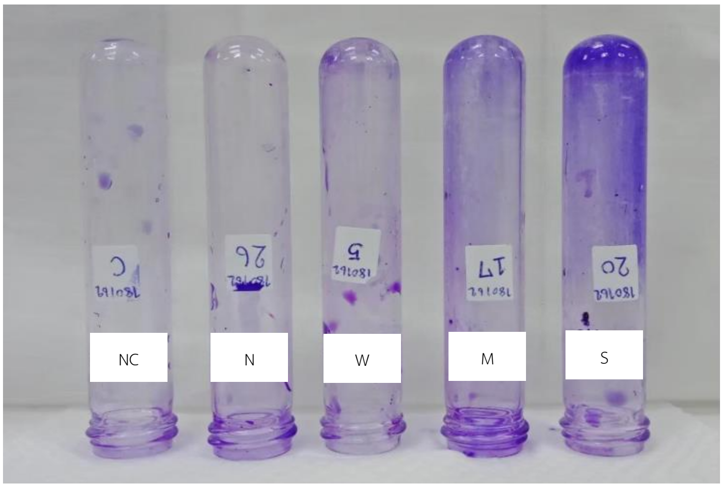


Figure 2 Coloring phenotypic of Biofilm on Tube method.
NC=Negative control, N=Non biofilm, W=Weak biofilm, M=Moderate biofilm, S=Strong biofilm.

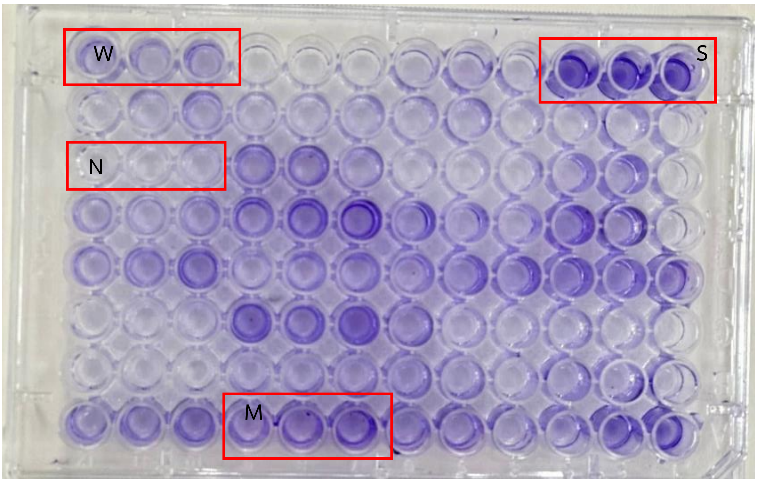


Figure 3 Color grading phenotypic of Biofilm on TCP method.
N=Non biofilm, W=Weak biofilm, M=Moderate biofilm, S=Strong biofilm.

Table 2 Grading of biofilm formation by different methods

Biofilm formation	TCP	TM	CRA
Strong	21 (17.79 %)	2 (1.69 %)	19 (16.10 %)
Moderate	25 (21.19 %)	30 (25.42 %)	-
Weak	30 (25.42 %)	50 (42.37 %)	62 (52.54 %)
Non-biofilm	42 (35.59 %)	36 (30.50 %)	37 (31.35 %)
Total	118	118	118

Table 3 Detection of biofilm, production among canine teeth by different method

Method	Total isolates	Biofilm production	Non biofilm production
TCP	118	46 (38.98 %)	72 (61.02 %)
TM	118	32 (27.12 %)	86 (72.88 %)
CRA	118	19 (16.10 %)	99 (83.90 %)

TCP= Tissue culture plate, TM=Tube method, CRA=Congo red agar

Table 4 Comparison of CRA with TCP for biofilm detection

CRA	TCP		Total
	Positive	Negative	
Positive	19 (*TP*)	0 (*FP*)	19
Negative	27 (*FN*)	72 (*TN*)	99
Total	46	72	118

TP - True positive. FP - False positive. FN - False negative. TN - True negative. N = TP + FP + FN + TN = 118. TPR = TP/N = 0.16. FPR = FP/N = 0. FNR = FN/N = 0.23. Apparent prevalence = TPR + FPR = 0.16 + 0 = 0.16. True prevalence = TPR + FNR = 0.16 + 0.23 = 0.39

Table 5 Comparison of TM with TCP for biofilm detection

TM	TCP		Total
	Positive	Negative	
Positive	32 (*TP*)	0 (*FP*)	32
Negative	14 (*FN*)	72 (*TN*)	86
Total	46	72	118

TP - True positive. FP - False positive. FN - False negative. TN - True negative. N = TP + FP + FN + TN = 118. TPR = TP/N = 0.27. FPR = FP/N = 0. FNR = FN/N = 0.12. Apparent prevalence = TPR + FPR = 0.27 + 0 = 0.27. True prevalence = TPR + FNR = 0.27 + 0.12 = 0.39

Table 6 Performance characteristics of TM and CRA for biofilm detection when compared with TCP

Method	Sensitivity	Specificity	Positive predictive value (PPV)	Negative predictive value (NPV)	Apparent prevalence (AP)	True prevalence (TP)
CRA	41 %	100 %	100 %	72 %	0.16	0.39
TM	69 %	100 %	100 %	83 %	0.27	0.39

Bacteria collected from dog teeth underwent comparison using three biofilm screening techniques. The results revealed that the TCP method surpassed the TM and CRA methods, consistent with the findings of Mohamed et al. (2016). TCP is recognized as a standardized test for detecting biofilm formation, aiding in classification, as noted by Triveda & Gomathi (2016). In the present study, 118 isolates were assessed for biofilm production using CRA, TM, and TCP. These phenotypic methods were easily and swiftly performed in most laboratory settings. CRA detected biofilm production in 19 isolates (16.10 %), TM in 32 isolates (27.12 %), while TCP demonstrated the highest biofilm production detection in 46 isolates (38.98 %). CRA and TM exhibited 100 % specificity, with TM (69 %) demonstrating higher sensitivity than CRA (41 %) compared to the TCP method. The TCP method is renowned for its effectiveness in detecting the most biofilms, making it the preferred choice due to its reliable sensitivity and specificity when contrasted with TM and CRA. The lower performance of the TM method compared to the TCP method may be attributed to observer variability in result interpretation or the use of plastic test tubes instead of glass, favoring the TCP method, as suggested by Saha et al. (2004). In contrast, the CRA method is known for its speed and easy of use. However, Rania et al. (2018) reported very low sensitivity (0.90 %) but a specificity of 97.40 % for biofilm detection, contradicting the findings of Oliveira and Oliveira & Cunha (2010) who reported over 89 % sensitivity and 100 % specificity.

The types of canine oral bacterial isolates have been reported, including both gram-positive bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes* group A, *Corynebacterium* spp., and *Lactobacillus* spp., as well as gram-negative bacteria such as *Shigella sonnei*, *Proteus penneri*, *Serratia liquefaciens*, *Klebsiella pneumonia*, *Achromobacter* spp., *Actinobacillus actinomycetemcomitans*, and *Pseudomonas* spp. The study results also revealed a higher % of infection in dogs with symptoms of gingivitis compared to dogs with a normal oral cavity (Phuket et al., 2016). *Actinobacillus actinomycetemcomitans* is identified as a significant factor in causing periodontitis, with diagnoses of aggressive periodontitis and chronic periodontitis supported by previous research (Dogan et al., 2003; Imbronito et al., 2008). However, in this study, the 118 isolates underwent detection of bacterial biofilm production using three different methods. While these methods are effective for detecting biofilm, the classification of bacterial biofilm requires confirmation through characterization methods, including biochemical tests and polymerase chain reaction (PCR) tests, in future studies.

This study summarized that the CRA test offers the advantages of being easier and faster to perform than other phenotypic tests for biofilm detection, with the added benefit of yielding viable colonies suitable for further studies. In contrast to the CRA, it can likely classify only strong biofilm formation (Sharvari & Chitra, 2012). It has also been reported that congo red directly interacts with the presented polysaccharide, forming colored complexes (Hassan et al., 2011). However, it exhibits a slight imprecision in identifying positive isolates when compared to TM analysis, showing lower sensitivity results than TM. In contrast, TCP continues to be the gold standard phenotypic method for detecting biofilm production, proving to be the most specific in this study. Upon comparing CRA and TM, it becomes evident that TCP provides a more accurate and quantitative approach to bacterial biofilm production. Therefore, TCP remains a viable screening technique for identifying biofilms in veterinary laboratories.

Conclusion

Bacterial biofilm production is a significant etiological factor in canine dental issues. Biofilm infections can result in chronic gingivitis and periodontal diseases with potentially severe outcomes. Therefore, detecting biofilm formation is crucial, as it allows for more informed antimicrobial choices by treating veterinarians. In this study, TM outperformed CRA in biofilm analysis, demonstrating superior sensitivity results. Among the three investigated phenotypic biofilm detection methods, the TCP method yielded the most effective results in isolating biofilms from canine teeth samples. These findings suggest that TCP can be employed to isolate biofilm-producing bacteria from canine teeth for laboratory purposes and may be adopted as a standard technique in animal health laboratories for separating microorganisms that produce biofilms. TCP proves to be a specific, sensitive and reproducible method for biofilm screening, potentially paving the way for the development of preventative and therapeutic products for biofilm-related diseases. In resource-constrained conditions, the CRA method and TM can be considered for biofilm detection, taking into account both rapidity and cost-effectiveness.

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Research article

Congo red, tube method and tissue culture plate method for detection of biofilm formation from canine teeth

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A B S T R A C T

A biofilm is a bacterial group that adheres to the canine tooth surface, known as plaque, which can cause canine gingivitis and periodontal disease. Contact with dogs or their saliva can transmit the biofilm to the dog's owner and other animals. This study aims to detect biofilm-producing bacteria and evaluate the suitability of three methods: Congo red agar (CRA), Tube method (TM), and Tissue culture plate (TCP) in a screening test for isolating biofilms from canine teeth. A total of 46 (38.98 %) isolated canine teeth showed positive biofilm production by TCP, which was considered the gold standard for biofilm detection. When compared to TCP, CRA detected 19 biofilm producers (16.10 %) and 99 non-biofilm producers (83.90 %), with sensitivity and specificity of 41 % and 100 %, respectively. TM correctly identified 32 biofilm producers (27.12 %) and 86 non-biofilm producers (72.88 %), with sensitivity and specificity of 69 % and 100 %, respectively. Compared to the TM and CRA approaches, the TM was superior to CRA in biofilm detection and demonstrated better sensitivity results. Among the three investigated phenotypic biofilm detection methods, TCP was the most focused, trustworthy, and simple method for identifying biofilms. It may be employed routinely as a standard screening technique for discovering bacteria that produce biofilms in an animal diagnostic laboratory.

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