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Antibiofilm Activity and Binding Specificity of Polyclonal DNA Aptamers on *Staphylococcus aureus* and *Escherichia coli*

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Aptamers are short, chemically synthesized, single-stranded DNA or RNA oligonucleotides that fold into unique three-dimensional structures. In this study, we aim to determine the antibiofilm activity and binding specificity of the six polyclonal DNA aptamers (S15K3, S15K4, S15K6, S15K13, S15K15, and S15K20) on *Staphylococcus aureus* BPA-12 and *Escherichia coli* EPEC 4. Aptamer S15K6 showed the highest percentage of antibiofilm activity against *S. aureus* BPA-12 (37.4%) as shown by the lowest OD_{570} value of 0.313. Aptamer S15K20 showed the highest percentage of antibiofilm activity against *E. coli* EPEC 4 (15.4%) as shown by the lowest OD_{570} value of 0.515. Aptamers S15K13 and S15K20 showed antibiofilm activities against both *S. aureus* BPA-12 and *E. coli* EPEC4, and thus potentially have broad reactivity. Furthermore, based on the binding capacity and Kd values from our previous study, the binding specificity assay of selected polyclonal DNA aptamers (S15K3 and S15K15) against *S. aureus* BPA-12, *E. coli* EPEC 4, *S. aureus* BPA-6, *S. agalactiae*, *E. coli* MHA-6, and *Listeria monocytogenes* were performed using qPCR. Aptamers S15K3 and S15K15 showed specific binding to *S. aureus* BPA-12, *E. coli* EPEC 4, *S. aureus* BPA-6, and *S. agalactiae*, but could not bind to *E. coli* MHA-6 and *L. monocytogenes*. Therefore, this study showed that the polyclonal DNA aptamers have antibiofilm activity and were able to bind to *S. aureus* BPA-12 and *E. coli* EPEC 4 bacteria.

Keywords: DNA aptamer, antibiofilm, qPCR, 16S rRNA analysis

Introduction

The common bacteria causing mastitis in dairy farms are *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Escherichia coli* [1, 2]. *S. aureus* and *S. agalactiae* are contagious pathogens that cause mastitis in livestock. *S. aureus* is the main cause of clinical mastitis and subclinical mastitis in dairy cattle in many countries [3, 4]. *S. aureus* colonizes on the nipples, milking hands and udders of infected cows. *S. aureus* also produces a toxin that is heat stable and remain active in pasteurized milk [5, 6]. *S. agalactiae* known as Group B Streptococcus (GBS) in the Lancefield classification can cause mastitis in dairy cattle [7]. Infection of the bacterial pathogen *S. agalactiae* is highly contagious [8]. Transmission

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between animals in herds occurs due to the lack of hygiene in the milking process, resulting in contamination of hands and milking equipment [9]. The digestive tract of animals and the environment are the reservoir of *S. agalactiae* [10]. Transmission of *S. agalactiae* goes through two cycles, namely the contagious cycle through milking machines and the fecal-oral cycle through drinking water [11].

E. coli is one of the main pathogens associated with environmental mastitis which cause acute systemic disease [12]. *E. coli* is common bacteria that mostly present in the digestive tract of animals. However, pathogenic *E. coli* can cause diarrhea in animals [13]. The identification of the difference between commensal and pathogenic *E. coli* strains lies in the presence of specific virulence factors [14]. *E. coli* causing mastitis can adapt in the udder thus the infection is persistent [15]. *E. coli* can be found in livestock manure and the exposure occurs due to dirty cage bottoms [16]. *E. coli* infection can occur at any time during lactation and the dry season [17].

Gram-positive bacteria (*S. aureus* and *L. monocytogenes*) and gram-negative bacteria (*E. coli*) have been isolated from cow's milk in various countries [18, 19]. *L. monocytogenes* is a pathogenic bacteria that causes the listeriosis disease in various animal species [18, 20]. Bovine mastitis caused by *L. monocytogenes* is less commonly reported in comparison with other mastitis pathogens [18, 21]. Infected animals with *L. monocytogenes* show clinical symptoms including encephalitis, septicemia, abortion, and diarrhea, but can also be asymptomatic and release *L. monocytogenes* in the feces [22]. *L. monocytogenes* can contaminate dairy products and is responsible for a foodborne disease in humans [20, 22].

Biofilm is a structured bacterial community attached to biotic and abiotic surface and produces exopolysaccharides that protect bacteria from antimicrobials, extreme environments, and increase antimicrobial resistance [23]. The formation of biofilms is one of the factors that contribute to antibiotic resistance and recurrence of mastitis infections [6, 24]. Aptamers are single-stranded DNA or RNA oligonucleotides that fold into unique three-dimensional structures to bind the target [25]. Aptamers were obtained by systematic evolution of ligands by exponential enrichment (SELEX) to the target through an in vitro selection process which included binding, separation, amplification and purification [26, 27]. Aptamer has been used as an inhibitor of several biofilm formation in bacteria [28, 29]. Aptamer can inhibit biofilm formation through the mechanism of forming secondary and tertiary structures attached to a specific target according to the shape of the aptamer [28].

This study aims to examine the antibiofilm activity of the six polyclonal DNA aptamers (S15K3, S15K4, S15K6, S15K13, S15K15, and S15K20). The six polyclonal DNA aptamers were obtained from in vitro selection against *S. aureus* BPA-12, *S. agalactiae*, and *E. coli* EPEC 4 [30]. The activity assay of six polyclonal DNA aptamers includes biofilms formation inhibition assay on *S. aureus* BPA-12 and *E. coli* EPEC 4, and binding specificity assay using qPCR on *S. aureus* BPA-12, *E. coli* EPEC 4, *S. agalactiae*, *S. aureus* BPA-6, *E. coli* MHA-6, and *L. monocytogenes*.

Materials and Methods

Bacterial strains and culture

S. aureus BPA-6, *S. aureus* BPA-12, *E. coli* EPEC 4, *E. coli* MHA 6, and *L. monocytogenes* were isolated from cow's milk with subclinical mastitis [31], while *S. agalactiae* was obtained from the Faculty of Veterinary Medicine, IPB University. The *E. coli* EPEC 4 strain was obtained from the previous study [31], and the strain has been molecularly characterized, however the data was unpublished. We also used this strain in our recent study [30]. All the bacteria used in this study have been molecularly characterized in our previous study [31], except *S. agalactiae* and *S. aureus* BPA-6. Therefore, it is necessary to perform 16S rRNA gene sequencing for their identification. *S. aureus* BPA-6, *S. aureus* BPA-12, *E. coli* EPEC 4, *E. coli* MHA 6, and *L. monocytogenes* were grown in NB medium, whereas *S. agalactiae* was grown in BHI medium.

Genomic DNA extraction

A total of 1.5 ml of the isolate was centrifuged at 11,000 ×*g* for 10 min at 4℃. The pellet was added with 540 µl of Tris-EDTA buffer and 10 µl of lysozyme, then mixed and incubated at 37℃ for 60 min. The mix was added with 200 µl SDS 10%, 100 µl NaCl 5 M, 80 µl 10% CTAB and incubated at 68℃ for 30 min. The chloroform was added 1:1 (v/v) and centrifuged 23,000 ×*g* for

10 min. The upper phase solution was moved into a new microtube, then added isopropanol with a volume ratio of 1:1 and centrifuged. The DNA pellet was mixed with 1 ml of cold 70% ethanol and inverted. The mixture was centrifuged at 10,000 ×*g* for 2 min at 4℃. The DNA pellets were air dried overnight. Dried DNA was dissolved in 27 µl of ddH_2O and 3 µl of RNAse. The DNA solution was incubated at 37℃ for 30 min, then stored at 4℃ [32].

16S rRNA PCR and sequencing analysis

The primers used for 16S rRNA PCR amplification were 8F (5'-AGAGTTTGATCATGGCTCAG-3') and 16R (5'-AAGGAGGTGATCCAACCGCA-3'). The PCR mixture consists of ddH_2O 6.4 µl, MyTaq DNA Polymerase 0.2 µl, 5 × MyTaq buffer 2 µl, primers 8F 0.2 µl, primers 16R 0.2 µl, DNA template 1 µl with total volume of 10 µl. The PCR process conditions were 95℃ 5 min; 35 cycles consisting of 95℃ 1 min, 55℃ 3 min, and 72℃ 1 min; and 72℃ 7 min. The PCR products were analyzed using 1% (w/v) agarose gel electrophoresis with 100 bp DNA Ladder marker. Ethidium bromide was used for staining and the bands were visualized under a UV transilluminator. The amplified 16S rRNA gene was directly sequenced and analyzed using Basic Local Alignment Search Tool (BLAST) in the NCBI program. The phylogenetic trees were created from the nucleotide BLAST results using Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Biofilm formation analysis and antibiofilm activity assay

The sequences and secondary structure of six polyclonal DNA aptamers (S15K3, S15K4, S15K6, S15K13, S15K15, and S15K20) used in this study have been published [30]. The crystal violet (CV) method was used to analyze the ability of *S. aureus*, *S. agalactiae*, and *E. coli*, to form biofilm. The bacteria were grown and incubated at 37℃ for 16 h [33]. The total volume of 100 µl were inserted into 96-well plate consisting of 1 µl bacterial culture and 99 µl medium. The 96-well plate was incubated at 37℃ for 24 h. The planktonic bacteria and medium were removed from the plate and washed using ddH_2O. The plate containing biofilm was added with 1% CV and incubated at room temperature for 15 min. The CV staining was washed 3 times using ddH_2O before it was drained. The CV fixation was carried out using 30% acetic acid and the plate was incubated at room temperature for 15 min. The absorbance was measured at 570 nm (OD_{570}) using a microplate reader [34]. The NB medium was used as negative control. Analysis of antibiofilm activity against *E. coli* EPEC 4 and *S. aureus* BPA-12 were performed using six polyclonal DNA aptamers with a concentration of 0.5 µM which were added to the bacterial culture test on 96-well plates. The results were represented as percentage of inhibition according to the following equation (ODc – ODt/ODc) × 100 where ODc = Optic density of negative control wells, ODt = Optic density of treated wells [35]. Data were analyzed statistical using GraphPad. The results demonstrated the means ± standard deviation (SD). To detect significant differences between treatment and control, one way ANOVA were used with significance at 5% ($p < 0.05$) level.

Aptamer binding specificity assay by qPCR

The bacterial cells (10^8) were prepared and incubated with 200 nM aptamers (S15K3, and S15K13) for 45 min at 30℃ under constant agitation of 220 rpm and were vortexed every 15 min. The mixture was centrifuged at 8,000 ×*g* for 6 min. The pellet was washed with 500 µl selection buffer (PBS + 1.4 mM $MgCl_2$). Centrifugation at 8,000 rpm for 6 min, was used for pellet separation. The pellet were mixed with 100 µl of ddH_2O and heated at 95℃ for 5 min to elute bound aptamers. The mixture was centrifuged at 13,000 ×*g* for 10 min at 20℃ to harvest bound aptamers in the supernatant. The quantification

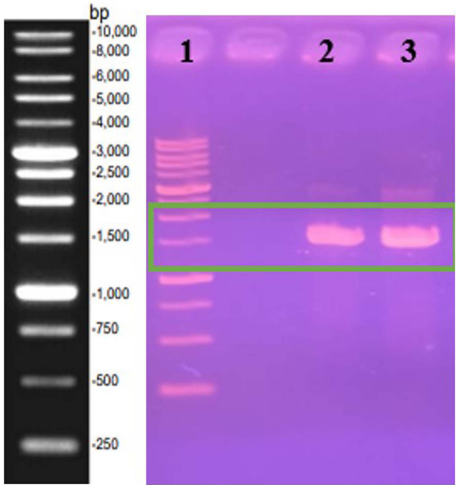

Fig. 1. Electrophoregram of 16S rRNA amplicons for *S. aureus* BPA-6 and *S. agalactiae*. 1. Marker; 2. *S. aureus* BPA-6; 3. *S. agalactiae*.

cycle (Cq) value of aptamers bound to the cells was carried out by qPCR analysis using SYBR Green. Ten µl qPCR reaction was set up containing 1 µl template (bound aptamers), 5 µl of SYBR green qPCR master mix, 0.12 µl forward primer, 0.12 µl reverse primer, and 3.76 µl of ddH_2O. The qPCR conditions were 95℃ 5 min; 20 cycles consisting of 94℃ 45 sec, 64℃ 45 sec, and 72℃ 45 sec; and 72℃ 8 min.

Results

Genomic DNA extraction, 16S rRNA PCR and sequencing analysis

The genomic DNA were quantified using Nanophotometer. The concentration of DNA genome of *S. aureus* BPA-6 and *S. agalactiae* were 1187.7 ng/µl and 608.1 ng/µl respectively. The 16S rRNA PCR method is commonly used to detect and identify pathogenic bacteria. The 16S rRNA PCR using primers 8F and 16R showed DNA bands of 1500 base pairs in size (Fig. 1). The 16S rRNA gene sequences of the two bacteria were compared with the NCBI sequence database. The two bacterial sequences were identified as ribosomal RNA strain of *S. aureus and S. agalactiae* with the intense identity of closely related data around 73–98% and 90–91%, respectively. According to the BLAST analysis, the E-values of the two strains were 0. The BLAST tree views of *S. aureus* and *S. agalactiae* were shown in Fig. 2 and Fig. 3, respectively.

Biofilm formation analysis and antibiofilm activity assay

The ability of *S. aureus* BPA-12, *E. coli* EPEC 4 and *S. agalactiae*, to develop biofilms were tested in vitro. *S. aureus* BPA-12 and *E. coli* formed a visible purple rings at the interface layer between the air and medium that attached on the 96-well plate which indicate the ability to form biofilms in vitro. *S. agalactiae* was unable to form biofilm since there was no visible violet ring (Fig. 4). Therefore, six polyclonal DNA aptamers (S15K3, S15K4, S15K6, S15K13, S15K15, and S15K20) were tested for their potential as antibiofilms against *S. aureus* BPA-12 and *E. coli* EPEC 4 (Fig. 5). Our preliminary study of aptamers at lower concentrations 0.1 µM showed antibiofilm activity against *S. aureus* BPA-12 and *E. coli* EPEC 4 bacteria. In this study, the concentration of aptamer was increased to 0.5 µM and the

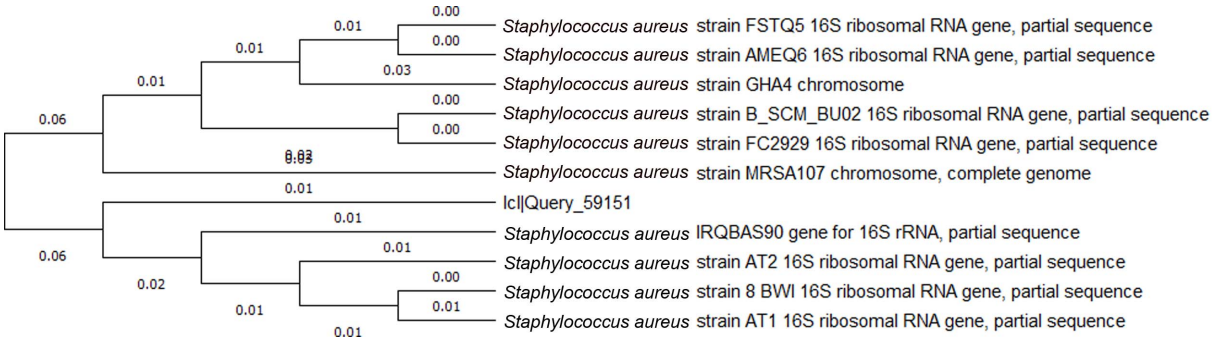

Fig. 2. Phylogenetic tree of 16S rRNA bacterial sequences of *S. aureus* BPA-6.

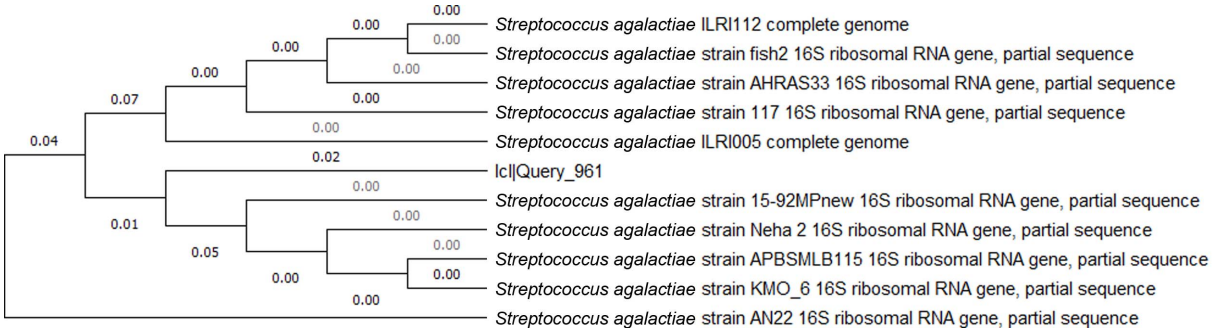

Fig. 3. Phylogenetic tree of 16S rRNA bacterial sequences of *S. agalactiae*.

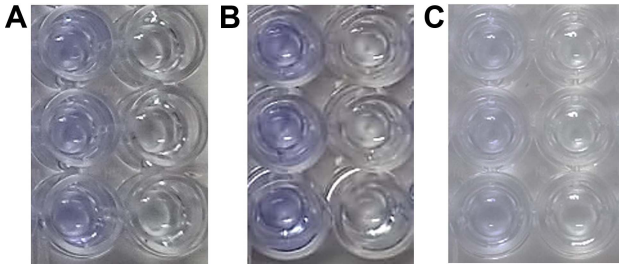

Fig. 4. The formation of biofilms in vitro. (A) *S. aureus* BPA-12. (B) *E. coli* EPEC 4. (C) *S. agalactiae*. Ring-like structures with violet colour on 96-well plate indicate the formation of biofilms. NB medium was used as negative control (N).

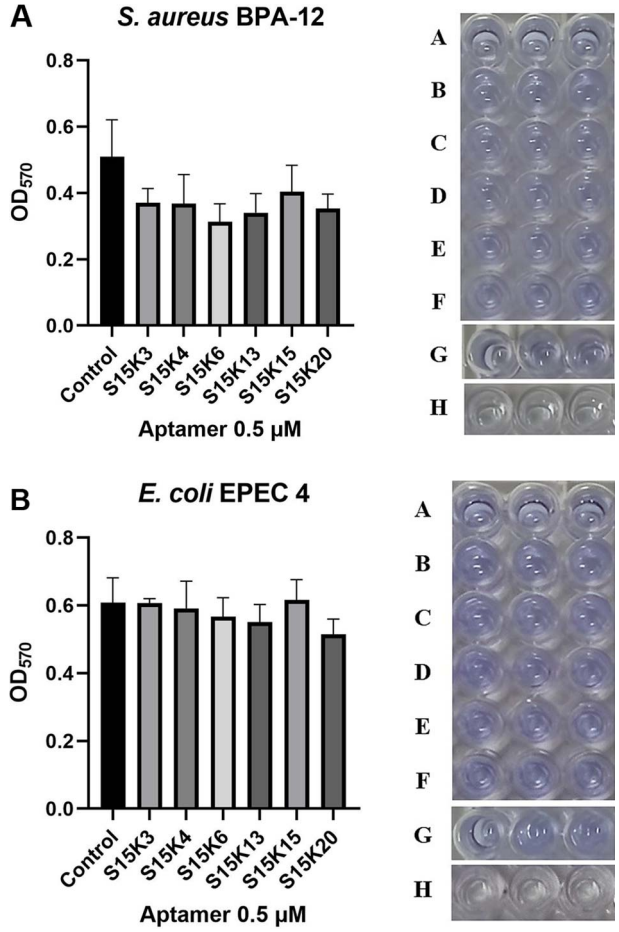

Fig. 5. Screening of antibiofilm activities of aptamers. Evaluation of biofilm inhibition quantitively on (A). *S. aureus* BPA-12 and (B). *E. coli* EPEC 4, by measuring the decrease of violet colour at OD_{570}. A: Aptamer S15K3; B: Aptamer S15K4; C: Aptamer S15K6; D: Aptamer S15K13; E: Aptamer S15K15; F: Aptamer S15K20; G: Control without aptamer; H: Control without bacteria.

results showed increasing biofilm activity against both of bacteria.

Among six aptamers tested, aptamer S15K6 showed the highest biofilm inhibition against *S. aureus* BPA-12 indicated by the lowest OD_{570} value of 0.313 (Fig. 5A) and the highest percentage of inhibition of 37.4% (Table 1). Aptamer S15K3, S15K4, S15K13, and S15K20 also showed strong inhibition percentage on *S. aureus* BPA-12 (Table 1). Aptamer S15K20 showed the highest biofilm inhibition against *E. coli* EPEC 4 indicated by the lowest OD_{570} value of 0.515 (Fig. 5B) and the highest percentage of inhibition of 15.4% (Table 1). Aptamer S15K15 was unable to inhibit biofilm formation on *E. coli* EPEC 4 (Fig. 5B). Aptamers S15K13 and S15K20 showed antibiofilm activities against *S. aureus* and *E. coli*, thus potentially have broad reactivity against both bacteria (Fig. 5). The statistical analysis using one way ANOVA showed antibiofilm activity of the polyclonal DNA aptamer against *E. coli* EPEC 4 did not show any significant different between treatments and controls ($p > 0.05$). Antibiofilm activity of the polyclonal DNA aptamer against *S. aureus* BPA-12 showed significant different ($p < 0.05$) between control vs S15K4, control vs S15K6, control vs S15K13, and control vs S15K20.

Aptamer binding specificity assay by qPCR

The binding specificity assay of selected polyclonal DNA aptamers (S15K3 and S15K15) against *S. agalactiae*, *E. coli* EPEC 4, *S. aureus* BPA-12, *L. monocytogenes*, *S. aureus* BPA-6, and *E. coli* MHA-6 were performed using qPCR method (Fig. 6). The polyclonal DNA aptamer S15K3 and S15K15 were selected because they have high binding ability and high affinity for *S. agalactiae*, *E. coli* EPEC 4, and *S. aureus* BPA-12 bacteria in previous studies. The polyclonal DNA aptamers (S15K3 and

Table 1. The inhibitory effect of DNA Aptamers on *S. aureus* BPA-12 and *E. coli* EPEC 4.

Aptamer	Inhibition Percentage (%)	
	S. aureus BPA-12	*E. coli* EPEC 4
S15K3	25.8	0.3
S15K4	26.3	2.8
S15K6	37.4	6.8
S15K13	31.8	9.4
S15K15	19.1	-1.3
S15K20	29.4	15.4

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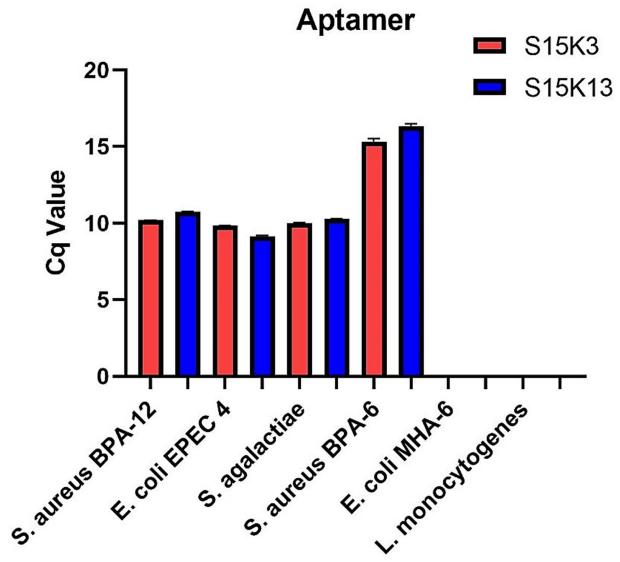

Fig. 6. The qPCR results of polyclonal DNA aptamers (S15K3 and S15K15) against the target pathogenic bacteria.

S15K15) were able to bind to *S. agalactiae*, *E. coli* EPEC 4, *S. aureus* BPA-12, and *S. aureus* BPA-6, but could not bind to *L. monocytogenes* and *E. coli* MHA-6. The Cq values of the two polyclonal DNA aptamers against *S. agalactiae*, *E. coli* EPEC 4, and *S. aureus* BPA-12 were approximately 10 which indicate that both aptamers have high binding ability to the three target bacteria. The two polyclonal DNA aptamers (S15K3 and S15K15) were still able to bind *S. aureus* BPA-6 even at Cq values of 15.32 and 16.32. The two polyclonal DNA aptamers could not bind to *L. monocytogenes* and *E. coli* MHA 6 as indicated by undetectable Cq values.

Discussion

S. aureus is often found and isolated in cases of mastitis in cattle [36]. Several virulence factors produced by *S. aureus* play an important role in disease pathogenesis including surface antigens (clumping factor A/ClfA), clumping factor B/ClfB), fibronectin binding protein A, collagen binding protein, elastin protein, sialoprotein, protein A/IgG-binding protein), degradation enzymes (serine protease/SpIA, serine V8 protease) and superantigenic toxins (leucocidin, enterotoxin, exfoliative toxin, and hemolysin) [37]. *S. agalactiae* is commonly found in cattle associated with clinical and subclinical mastitis [7, 38]. *S. agalactiae* can cause persistent infections with relatively low cure rates [6, 39]. The virulence factors of *S. agalactiae* include FbsA protein (fibrinogen-binding protein A), HlyB protein (hyaluronate lyase B) and capsule polysaccharides [10, 40]. The FbsA protein allows *S. agalactiae* to bind to fibrinogen and mobilize the host extracellular matrix, and plays a role in the protective mechanism of the immune system, preventing opsonization by macrophages and neutrophils [40]. The function of HlyB protein in the pathogenesis of *S. agalactiae* is to degrade polysaccharides and support the spread of *S. agalactiae* infection [8, 41]. The polysaccharide capsule located on the cell membrane allows *S. agalactiae* to infect the host and escape the immune system, supports bacterial adhesion to epithelial surfaces, and inhibits phagocytosis by macrophages and neutrophils [8].

Mastitis caused by *E. coli* is usually sporadic with clinical symptoms that vary from mild to severe [16]. Several *E. coli* virulence factors related to pathogenicity include toxins, invasins, adhesins, capsule production, iron scavenging, and ability to resist serum complement [42]. The mechanism of adaptation of *E. coli* in the udder is related to the extracellular matrix structure of polysaccharides, flagella and pili [43]. Some strains of *E. coli* can adhere and internalize into udder epithelial cells so that the pathogen is persistent in the tissue and causes recurrent cases of mastitis [44]. Controlling of *E. coli* infection is carried out by maintaining the cleanliness of the cage and the environment as well as applying correct milking procedures [42].

The ability of bacteria to form biofilms is an important virulence factor in the pathogenesis of mastitis [45]. The presence of biofilm in the udder causes a decrease effectiveness of antibiotics so it is difficult to treat and the infection becomes persistent [46]. *S. aureus* is pathogenic bacteria that causes mastitis that can quickly attack all types of cells in the udder, making it difficult to control [47]. *S. aureus* infection can be chronic due to the ability of *S. aureus* to form biofilms [48, 49]. The biofilm on *S. aureus* develops antibiotics resistance and escape the host phagocytic mechanism so that the infection can be persistent [5, 50]. *S. agalactiae* as a mastitis pathogen which also can form biofilms [51]. The presence of pili on the surface of *S. agalactiae* helps in the attachment process and biofilm formation [52]. *E. coli* is an environmental pathogen that can cause mastitis [28]. The ability of biofilm formation in *E. coli* is related to

fimbriae and the production of exopolysaccharides including colanic acid (M antigen), cellulose, β-1,6-N-acetyl-D-glucosamine (PGA) [44].

Aptamers have interactions on the active site of the target to reveal the mechanisms of aptamers [53]. One of the mechanisms of aptamers in reducing the growth of pathogenic bacteria is through the formation of antibiofilm [54]. Aptamers inhibit bacterial growth through direct binding of the aptamers to cellular membrane components [55]. Flagella are one of the targets of aptamer attachment so that it inhibits the formation of biofilms [28]. Flagella are used by bacteria to attach to a surface so that they can form a biofilm [56, 57]. Aptamers which attached to flagella limit the bacteria movement, thus inhibiting bacteria from forming mature biofilms [28]. Antibiofilm activity assay of six types of aptamers against *E. coli* EPEC K1.1 showed different activities [33]. Investigate of six DNA aptamers bound to *S. aureus* cells in the biofilm showed one aptamer that could facilitate the accumulation of liposomes around *S. aureus* cells inside the biofilm [58].

Specificity is the selective binding of aptamer to the chosen target [59, 60]. Investigate of several aptamers showed varying target binding efficiencies against different strains of *Pseudomonas aeruginosa* [61]. Binding specificity assay is required to minimize false positive results [60]. Aptamers need to demonstrate high affinity, avidity, specificity for downstream applications [60, 62]. Affinity and specificity are important parameters for diagnostic applications [60]. Aptamer binding ability is affected by the structure and binding affinities of the aptamer [63, 64]. To increase the binding affinity of aptamers, several approaches can be used, including optimization of the aptamer sequence; stabilization of the aptamer structure; introduction of the hydrophobic moiety into the aptamer; and conjugation of binding motifs [64]. The increase in affinity, avidity and specificity of the aptamer that directly binds to the target will increase the specificity and sensitivity of the test [60].

The screening of antibiofilm assay showed that aptamer S15K6 and S15K20 has the highest antibiofilm activities against *S. aureus* BPA-12 and *E. coli* EPEC 4, respectively. Whereas aptamers S15K13 and S15K20 showed the ability to inhibit the formation of biofilms against both *S. aureus* BPA-12 and *E. coli* EPEC 4. The binding specificity assay of polyclonal DNA aptamers (S15K3 and S15K15) showed that they were able to bind *S. agalactiae*, *E. coli* EPEC 4, *S. aureus* BPA-12, *S. aureus* BPA-6, and but could not bind to *E. coli* MHA-6 and *L. monocytogenes*.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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