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ORIGINAL ARTICLE

Gold nanoparticles (AuNP)-based aptasensor for enteropathogenic *Escherichia coli* detection

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Abstract

Background Diarrhea is a major cause of severe gastrointestinal illness in the infant especially in many developing countries. Although this molecular technique has been accepted as standard technique to detect Diarrhea-causing EPEC, the practical aspect of this technique for in-site rapid screening purposes is still facing a major challenge. In this study, we characterized EPEC specific aptamers and applied it as an AuNP-based aptasensor for point of care (POC) diagnosis purpose.

Methods As many as six selected DNA aptamers was screened using target bacteria and the bound aptamer was measured by qPCR technique. Moreover, Kd value for each optimal bound aptamer was measured by using the same technique. Colorimetry assay was applied to test specificity and LOD of AuNP-based aptasensor.

Results Two DNA aptamers have been successfully obtained to detect Enteropathogenic *Escherichia coli* K.1.1. DNA aptamer S8-7 exhibited constant dissociation (Kd) value of 17.08 nM, while DNA aptamer S10-5 exhibited Kd value of 34.14 nM. AuNP-based aptasensor showed high selectivity and specificity for EPEC K.1.1 with a limit of detection (LOD) value of 10^5 CFU/mL. Truncation study on DNA aptamer S8-7 showed that elimination of primer binding sequence only slightly increased both performance of detection and LOD value of AuNP-based aptasensor.

Conclusion Further study is necessary to improve AuNP-aptasensor performance such as through mutagenesis approach on targeted DNA aptamers before AuNP-based aptasensor can be applied as a biosensor in point of care (POC) diagnosis.

Keywords DNA aptamer · EPEC · Aptamer Truncation · AuNP-based aptasensor

Introduction

Escherichia coli has been considered to be the very first bacterial species that inhabits a human's intestinal gut. This non-virulent commensal microbe immediately colonizes the infant's gastrointestinal tract after birth, living harmlessly in the intestines and rarely causing disease in healthy individuals [1, 2]. However, other strains of *E. coli* that cause severe diseases such as diarrhea, sepsis/meningitis, and urinary tract infections in humans also exist. They can infect both healthy and immune-compromised individuals. These pathogenic characteristics of *E. coli* have been obtained from other bacterial species due to the horizontal transfer of virulence factor genes [3].

Diarrhea is still becoming an overwhelming problem in many developing countries where clean water, poor sanitation, and malnutrition are inadequate [4]. It is a major cause of severe gastrointestinal illness in the infant but it can also occur in older children, adolescents, and adults [5, 6]. Diarrheagenic *E. coli* (DEC) is one of the etiologic agent for diarrheal illness where this type of *E.coli* is classified into six categories based on their pathotypes named enterohemorrhagic *E.coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC), respectively [7, 8]. Furthermore, diarrheagenic *E. coli* is further divided based on the ability of antibodies to distinguish a panel of surface O (somatic),

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H (flagellar), and K (capsular) antigen so called serotyping, a classical method to define diarrheagenic *E. coli* to be associated with certain clinical syndromes [9, 10]. Among diarrheagenic *E. coli* types known today, EPEC is the most common *E. coli* found in children with acute diarrhea [11, 12]. Interestingly, based on recent findings, it is suggested that two types of EPEC exist (typical EPEC and atypical EPEC). They could be discriminated based on the presence or absence of the bfpA gene in the *E. coli* adherence factor plasmid (EAF), the bundle-forming pili encoding gene. Atypical EPEC with this bfpA gene plays a significant effect in both pediatric endemic diarrhea and diarrhea outbreaks worldwide [13, 14]. Moreover, the genome plasticity of EPEC makes this pathogenic bacteria troublesome to some existing antibiotics such as ampicillin, streptomycin, piperacillin, cephalosporins, and carbapenems, warming the need for early preventive diagnosis in suspected reservoirs or unhygienic foods, and environment to prevent sporadic and outbreak-associated diarrhea in community [6, 15, 16].

Molecular detection using PCR method and its modification has been widely used to identify EPEC in clinical specimens (stools and blood) of hospitalized individuals with diarrhea or in other samples (water, foods, and feeds) that are considered as EPEC reservoirs, replacing current serotypes-based microbiological approach that is insufficiently to differentiate DEC from nonpathogenic *E. coli.* Although this molecular technique have been accepted as a standard procedure to detect EPEC in most clinical laboratory settings and claimed to have 100% correctly in identifying the EPEC, yet the practical aspect of this technique for in-site rapid screening purposes is still facing a major challenge until recently such as (1) waiting time in obtaining the diagnosis result and (2) the need of trained skill to both formulate complicated PCR reagents and to operate the PCR machine [17, 18]. On the other hand, aptamers, an emerging technology with a great promise as chemical antibody for rapid diagnosis has attracted many researchers worldwide to be applied as a part of biosensor component for clinically important *E. coli* detection [19]. Various aptasensor formats for this pathogenic *E. coli* detection have been fabricated using either electrochemical or optical principle which targets whole cells of *E. coli* or its biological components [20, 21].

Aptamers are single strand oligonucleotides either as RNA or DNA obtained through targeting target with chemically synthesis of sort oligonucleotides library by technique called systematic evolution of ligands by exponential enrichment (SELEX) which is iterative process of binding, separation, amplification and purification [22, 23]**.** Aptamers produced by SELEX technology mostly exhibits excellent characteristic in term of specificity, sensitivity, and stability and in contrast to antibody, aptamers are very easy to be chemically modified to increase their performance and applicability and also thanks to this outstanding technique, aptamers discovery for *E. coli* detection have tremendous effect in medical, environment, food safety and agriculture fields [24–26]**.** In previous study, we have isolated DNA aptamers using bacterial-SELEX against EPEC K.1.1 as target selection. DNA aptamers that were successfully isolated showed diversity in terms of their secondary structure and G-quadruplex within sequence. Moreover, melting curve analysis result of DNA aptamers provided important information for its application as a diagnostic tool for EPEC detection [27].

Therefore, the aims of this study were (1) to characterize DNA aptamers that specifically bind to the EPEC isolated from young children with diarrheal disease in West Java region, Indonesia and (2) to develop AuNP-based aptasensor using selected DNA aptamers as an attempt to evaluate these potential DNA aptamers to be used as part of biosensor component in point of care (POC) diagnosis.

Material and methods

Bacterial strains and culture conditions

EPEC K.1.1 was used as the target strain and grown on Luria–Bertani (LB) medium at 37 °C. *Lactobacillus plantarum* was used as negative selection in screening test of DNA aptamer candidates and growth in De Man, Rogosa and Sharpe (MRS) medium at 37 °C. Other strain, *E.Coli* DH5α and *E.Coli* TOP10 were also used in this study for the specificity test and each *E.coli* was grown in LB medium at 37 °C. In addition, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella typhi* were grown in Nutrient broth at 37 °C also used for specificity test purpose. Except for EPEC K.1.1, all bacteria used in this study are from collection of the Research Center for Genetic Engineering, National Research and Innovation Agency (BRIN), Cibinong, Bogor.

DNA aptamers screening using qPCR method

In pair, single colony of EPEC K.1.1 and *L. plantarum* were cultured in defined medium until McFarland standard optical density (OD) at 600 nm reached 0.3 (equivalent to ~ 10^8 CFU/mL). Following that, 1 mL of culture was collected by centrifugation at 8000 rpm for 6 min. The pellets were washed twice with 1 × phosphate buffered saline (PBS) and centrifuged at the same speed for 1 min. Furthermore, 100 nM DNA aptamer was added to the pellet in 1 × PBS containing 1.4 mM $MgCl_2$ (selection buffer), followed by incubation for 45 min at room temperature with constant agitation at 220 rpm. The aptamer-bacteria complex was collected by centrifugation at 8000 rpm for 6 min, followed by

washing using 1 mL selection buffer. The solution was centrifuged again at 8000 rpm for 6 min to remove the unbound primer. The aptamer-bacteria complex was resuspended in 100 µl of double-distilled water (ddH_2O) and the bound aptamer was collected by heating the suspension at 95 °C for 5 min, followed by centrifugation at 12.000 rpm for 10 min at 20 °C. The solution from this centrifugation step which contains aptamers was used as a template in qPCR reaction. Each of qPCR reaction contained 5 µl of qPCR solution (SYBR® Green Realtime PCR Master Mix, Toyobo), 0.4 µl of each forward primer (5′-CCGGAATTCCTAATA CGACTC-3′) and reverse primer (5′-CCGCGGCCGCGT TTTCAATA-3′), 1 µl of template, and nuclease-free water up to 10 µl. The qPCR conditions were as follows: initial heat activation at 95 °C for 5 min and 18 cycles of 94 °C for 45 s, 56.6 °C for 45 s, 72 °C for 45 s, and a final elongation step of 5 min at 72 °C. The Ct value was obtained in qPCR reaction used Rotor-Gene Q (Qiagen). Each qPCR sample was done in triplicate reaction.

Kd evaluation of selected DNA aptamers using qPCR method

Kd value of selected DNA aptamers was determined by qPCR as followed; DNA aptamer with different concentration (100 nM, 200 nM and 400 nM) was bound to 10^8 CFU/ml of EPEC K.1.1, incubated for 45 min at room temperature with shaking at 200 rpm. Bound aptamer was retrieved using protocol as discribed early, and this bound aptamer was used as template in qPCR reaction. Apparent Kd values for each aptamer were determined by Linewaver-Burk analysis followed method using the formula: $1/[\text{complex}] = Kd/[Cmax] \times 1/[\text{aptamer}] + 1/[Cmax]$, where Kd is the steady state dissociation constant, [Complex] is the concentration of the complex bacteria-aptamer, [Cmax] is the concentration of the complex at maximal binding capacity, where all the binding sites are occupied by the aptamer, and [aptamer] is the concentration of the aptamer [26].

Establishment of AuNP-based aptasensor for EPEC K.1.1 detection

For this purpose, we used 15 nm diameter of AuNP (stabilized suspension in citrate buffer with OD 1) purchased from Sigma Aldrich (777137-25ML). Optimization of NaCl concentration was obtained by adding salt with increasing concentration (0.0156; 0.0312; 0.0625; 0.125; 0.25; 0.5; and 1 M) into solution containing DNA aptamer (100 nM) and AuNP (62.5 µl) with a total volume of 200 µl. The solution was incubated for 10 min at room temperature, then color shift in the solution were scanned at a wavelength of 460–750 nm using Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific). In addition, the optimization of DNA aptamer concentration was performed by adding various concentrations of aptamer (50, 100, and 200 nM) to a solution containing optimized NaCl and AuNP (62.5 µl) with a total volume of 200 µl. The solution was incubated for 10 min at room temperature, then color shift in the solution were scanned using the same approach [28].

Specificity test of aptasensor to bacterial target EPEC K.1.1 and *E. coli* DH5α, *E. coli* TOP10, *S. typhi*, *S. aureus* and *L. monocytogenes* were assessed. Each bacterial cell was prepared with 10^8 CFU/mL with washing process done as previously described. Then, 50 µl of each bacterium was added into solution containing of 6.5 µl of AuNP, NaCl (0.0625 M), DNA aptamer (50 nM) at total volume of 200 µl. Solution was mixed well and incubated for 10 min. For LOD test, several number of EPEC K.1.1 was prepared (10^7 10^6 10^5, and 10^4 CFU/mL) and mixed each diluted bacterium with 62.5 µl of AuNP, NaCl (0.0625 M), DNA aptamer (50 nM) at total volume of 200 µl. This solution was stayed for 60 min, 120 min, and overnight incubation. The color change was measured at 620 nm using Varioskan™ LUX multimode microplate reader, Thermo Fisher Scientific [20, 29].

Result

DNA aptamer screening against EPEC K.1.1

A total of six DNA aptamers were selected from the bacterial-SELEX procedure in a previous study for their ability to specifically recognize EPEC K.1.1. Two DNA aptamers, DNA aptamer S8-7 and DNA aptamer S10-5, demonstrated the best binding capacity for EPEC K.1.1 out of the six DNA aptamers evaluated. These two exhibited the lowest Ct values among the DNA aptamers tested (Ct value < 10) (Fig. 1). On the other hand, no Ct value was observed on these two DNA aptamers when a template from eluent of *L. Plantarum*-bound DNA aptamer was used, confirming that our bacterial-SELEX technique which was developed in our previous study enable to isolate DNA aptamers with good specificity for EPEC K.1.1. Based on this finding, we selected DNA aptamer S8-7 and DNA aptamer S10-5 for further analysis.

Affinity assessment of selected DNA aptamers

DNA aptamer S8-7 and DNA aptamer S10-5 showed different Kd value against EPEC K.1.1 as measured by the binding saturation curve obtained from qPCR. As shown in Fig. 2, the Kd value of DNA aptamer S8-7 was 17.18 nM, while the Kd value of DNA aptamer S10-5 was 34.14 nM, respectively.

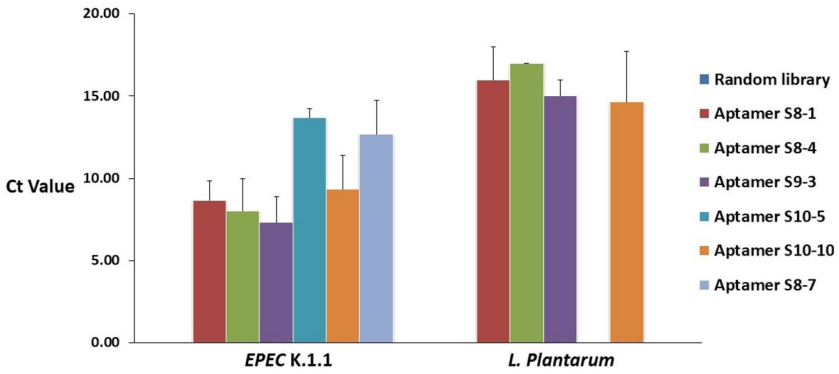

Fig. 1 qPCR-based screening of DNA aptamers to the target and its counterpart

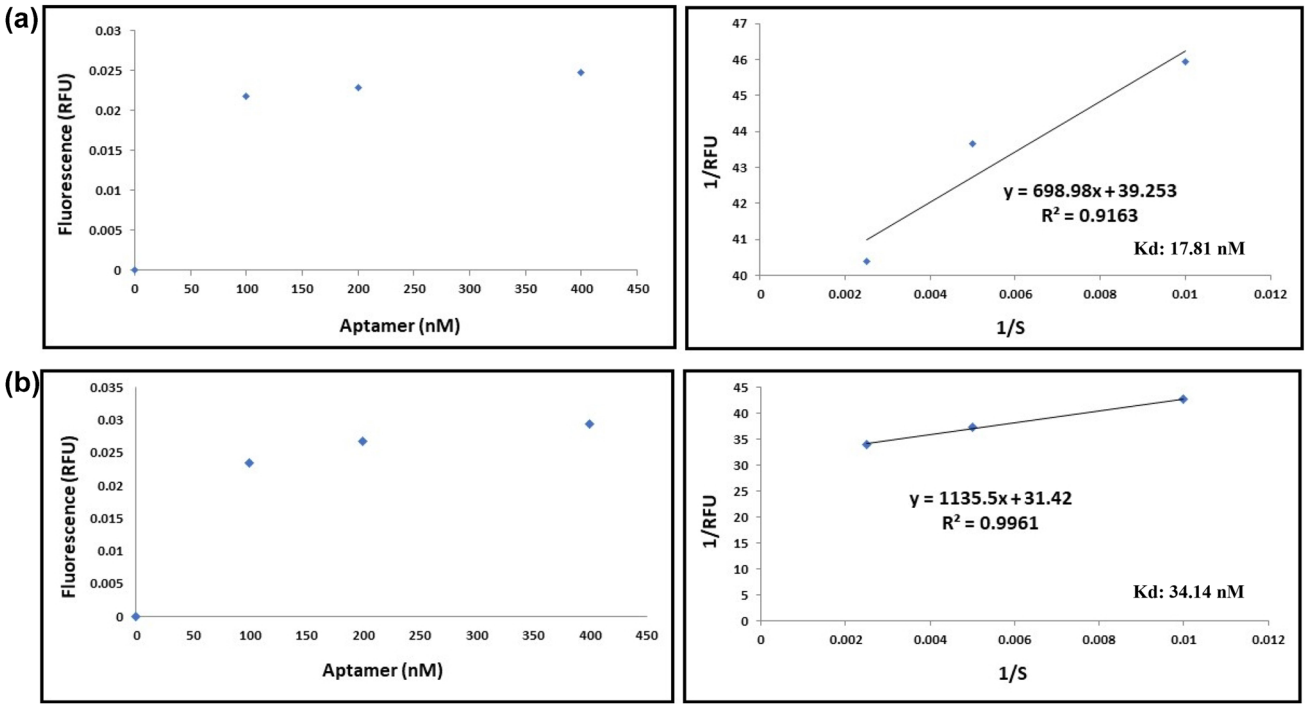

Fig. 2 Kd value determination of two selected DNA aptamers using binding affinity using qPCR. **A** binding affinity curve of DNA aptamer S8-7 with its kd value and **B** DNA aptamer S10-5 with its kd value

Parameters establishment for AuNP-based aptasensor for EPEC K.1.1 detection

NaCl and DNA aptamer with different concentration were tested to evaluate the effect of NaCl on color stability of AuNP-based aptasensor without the presence of target bacteria. From the range of NaCl concentration tested, 0.0625 M of NaCl was the best concentration in preserving the red color of AuNP-based aptasensor as observed at 520 nm (Supp. Fig. 1A and B). Meanwhile, a color sifting from red to red–purple was observed at 620 nm when NaCl concentration was increased up to 1 M. No such effect was observed when NaCl was replaced with DNA Aptamer even when highest concentration of DNA aptamer was applied (200 nM) (Supp. Fig. 1C and D).

Selectivity, specificity and LOD of AuNP-based aptasensor for EPEC K.1.1 detection

AuNP-based aptasensor in this study exhibited both high selectivity and specify for EPEC K.1.1 where AuNP-based aptasensor with DNA aptamer S8-7 showed better performance in detecting EPEC K.1.1 as indicated by the lower OD value for non-target bacteria, although slightly increased in the absorbance value at 620 nm for DNA aptamer S10-5 was observed for the target bacteria (Fig. 3). Furthermore, the best LOD of AuNP-based aptasensor was achieved at

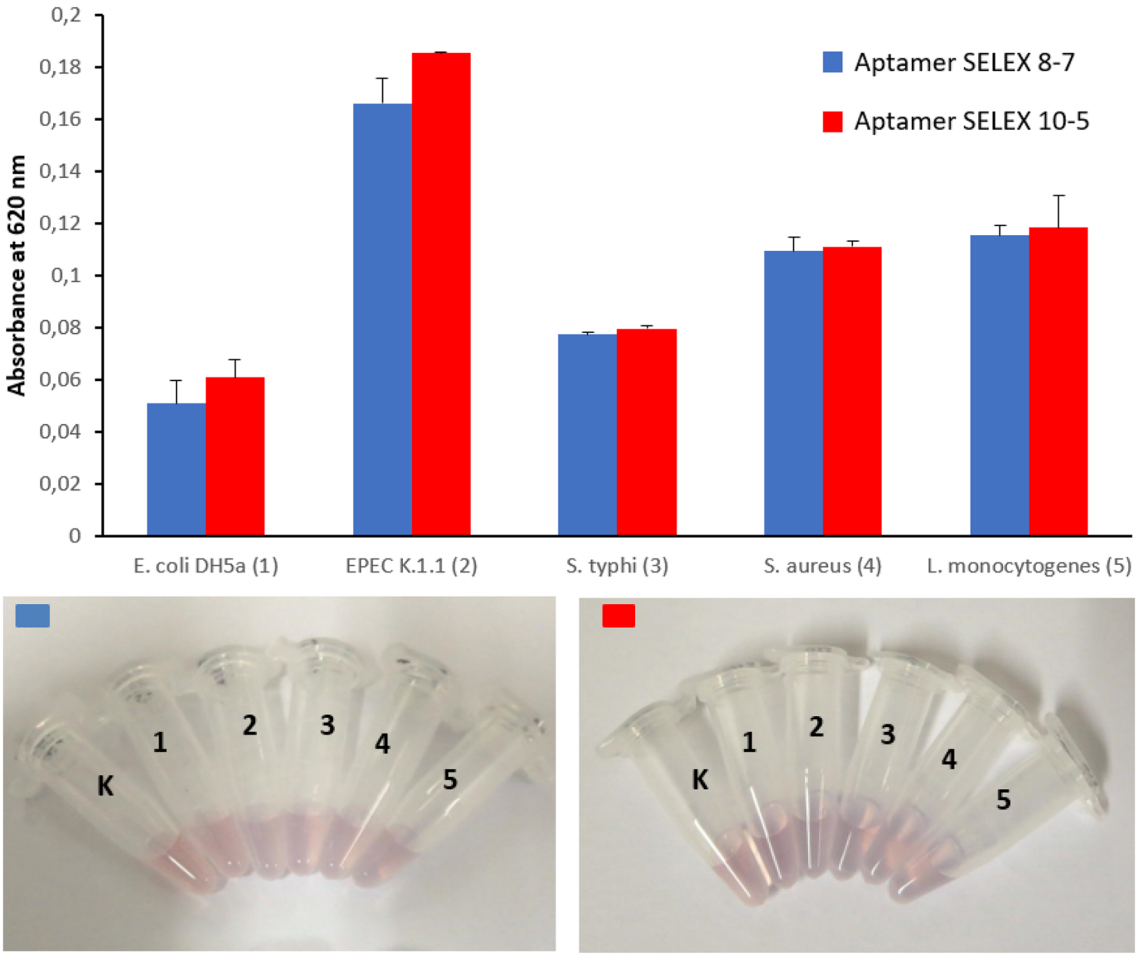

Fig. 3 Specificity test of AuNP-based colorimetric assay. DNA aptamer S8-7 and DNA aptamer S10-5 were used as bio-capturing agent to differ EPEC K.1.1 from several pathogenic bacterial species

overnight incubation with value 10^6 CFU/mL (Fig. 4). A body of evidence from other studies stated that some primer binding sequences within aptamer could create an internal stem-loop structure that can interfere the target-aptamer binding process, ultimately reducing the performance of aptamer [30]. In line with this statement, we hypothesized that the LOD value of our aptamers could be further reduced by eliminating the primer binding sequences from aptamers.

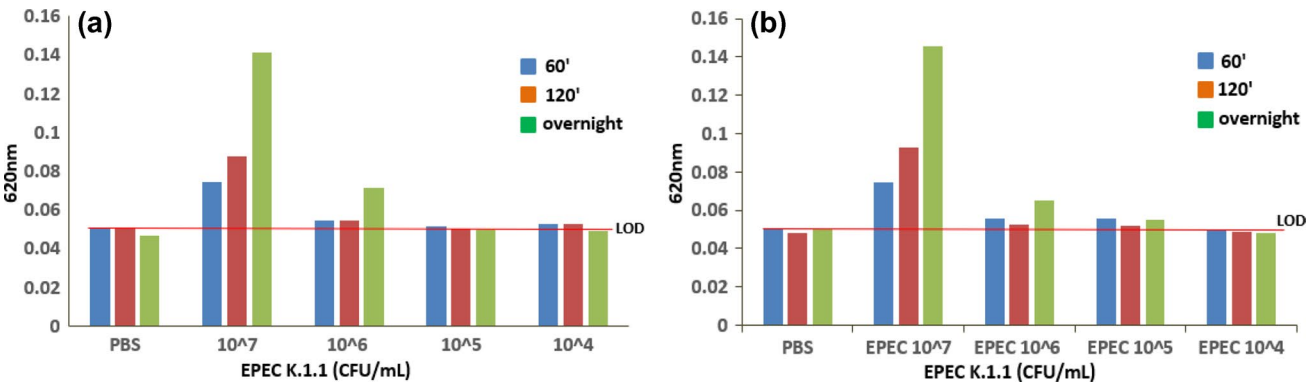

Fig. 4 Limit of detection test of AuNP-based colorimetric assay toward EPEC.K.1.1using **A** DNA aptamer S8-7 and **B** S10-5 was used as bio-capturing agent

Aptamer S8-7 in this study contained stem-loop structures at both primer binding sequences which might affect the binding affinity of aptamers (Fig. 5A). We applied truncation approach to eliminate this primer binding sequence either at 3-end (truncated DNA aptamer S8-7.1) or 5-end (truncated DNA aptamer S8-7.2) from original aptamer S8-7. Eliminating both sequence from DNA aptamer (DNA aptamer S8-7.3) slightly improved the specificity and specificity detection against EPEC.K1.1 compared to original DNA aptamer S8-7. Meanwhile, improvement was not observed when the primer binding sequence at 3-end was removed from DNA aptamer. However, it was observed when primer binding sequence at 5-end was eliminated from DNA aptamer S8-7 (Fig. 5B). Interestingly, DNA aptamer S8-7.2 has minor effect on improving the LOD value of AuNP-based aptasensor after overnight incubation (10^5 CFU/mL) (Fig. 5C).

Discussion

In this study, qPCR technique was chosen in screening the EPEC.K1.1 specific DNA aptamers due to its simple procedure, yet it is robust technique in term of quantifying the DNA aptamer in range of nanomolar scale [29, 31]. The same screening technique using qPCR in obtaining high binding affinity of DNA aptamers for clinical importance *E. coli* has also been reported elsewhere. For instance, DNA aptamers for nosocomial urinary tract infections associated *E. coli* and sepsis associated *E. coli* [26, 32]. The affinity of the aptamer is an essential measurable factor in evaluating the performance of the DNA aptamer towards its target. This parameter indicates the strength of interaction between an aptamer and its target as measured by the dissociation constant (Kd) [33]. The lower the Kd value of the aptamer, the stronger binding was with its target. Selected DNA aptamers in our study showed in nanomolar range which indicated strong binding affinity against EPEC.K.1.1. Kd value of DNA aptamer in our study was also comparable to other reported *E. coli* specific DNA aptamers regardless qPCR or non-qPCR coupled SELEX procedure had been selected [24, 26, 32, 34, 35] (Supp. Table 1).

In an attempt to establish a naked-eye detection of EPEC K.1.1 using an AuNP-based aptasensor, it is essential to know the color development of this platform as an indicator of the presence of the target. Based on a current finding, the readout of an AuNP-based aptasensor could be either a red (salt-induced AuNP aggregation) or blue (anti-AuNP

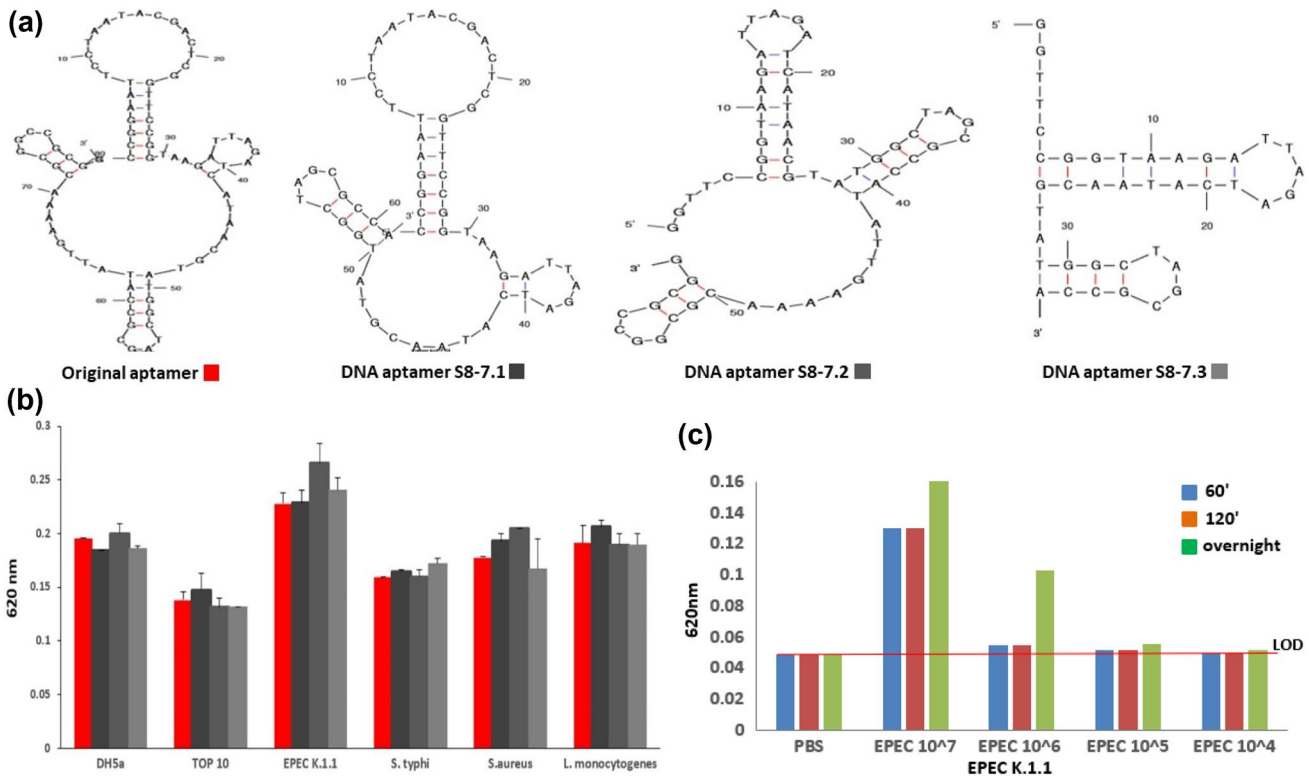

Fig. 5 Effect of DNA aptamer sequence truncation on AuNP-based aptasensor performance. **A** Truncation approach on DNA aptamer S8-7, **B** Specificity and selectivity test of DNA truncated aptamer S8-7.2, and **C** LOD test for DNA truncated aptamer S8-7.2 in AuNP-based colorimetry platform

aggregation) appearance. In particular, this color-shifting largely depends on the complex interaction among AuNP, aptamer, salt, and target in the solution [37, 38]. Among those substances, NaCl salt plays critical role in this regard and stable red color solution without the presence of bacterial target will dictate the unbiased diagnostic readout. NaCl salt significantly affected the color stability of AuNP-based aptasensor in our study as increasing the salt concentration result in a color shifting from 520 to 620 nm. No such event was observed when concentration of DNA aptamers was applied even at high concentration. CSC value was achieved at NaCl concentration of 0.0625 M: a salt concentration at which the dispersed AuNPs released solely from the AuNPs-aptamer complex not coming from the excessive salt added into the solution begin to precipitate and aggregate in the presence of its target, this aggregation process was marked by a color shifting from red to intense red–purple in solution [28, 39].

Another importance characteristics of aptasensor are selectivity and specificity of detection. In our study, this test was done by detecting bacterial target with other non-bacterial target such as *E. coli* DH5α, *E. coli* TOP10, *S. typhi*, *S. aureus*, and *L. monocytogenes* where the color shifting from red to blue is an indicator of the present of target in the solution. In this study, The AuNP-based aptasensor either using DNA aptamer S8-7 or S10-5 as bio-capturing agent exhibited both high selectivity and specify for EPEC K.1.1. Moreover, LOD value for AuNP-based aptasensor was significantly influenced by the incubation time where prolong incubation from 60 or 120 min to overnight incubation time reduced its LOD value by approximately 10 time (10^7 CFU/mL to 10^{76} CFU/mL). LOD value of AuNP-based aptasensor using original DNA aptamer in our study was slightly different compared to other *E. coli*-detecting aptasensor platforms. For instance, lateral flow biosensor exhibited LOD value of 10 CFU/mL, chemiluminescent aptasensor exhibited LOD value of 4.5×10^3 CFU/mL, and SERS aptasensor exhibited LOD value 10^2 CFU/mL [25, 40, 41]. It has been shown that DNA aptamers properties can be improved by eliminating either 5′-primer or 3′-primer binding sites from original aptamer [42]. In line with this statement, we observed that eliminating both primer binding sites improved AuNP-based aptasensor for EPEC.K1.1 detection. Moreover, removing 5′-primer binding sites from its original DNA aptamer significantly increased the performance of our AuNP-based aptasensor as proven by the decrease in LOD value by 10 time (10^6 CFU/mL from AuNP-based aptasensor using original DNA aptamer S8-7 to 10^5 CFU/mL), although this approach could not reach the comparable LOD value as observed in reported *E. coli*-detecting aptasensor platforms [25, 36]. Nonetheless, we predicted stem-loop structure within 5′-primer binding sites might prevent the optimal binding capacity of DNA aptamer to its target. Moreover, truncation approach in our study also indicated that there were specific DNA bases within stem-loop structure of DNA aptamer S8-7 that influenced the overall binding affinity of DNA aptamer towards its target, though further study is need to be conducted to uncover this bottleneck such as through mutagenesis approach.

Conclusion

Two DNA aptamers, DNA aptamer S8-7 and DNA aptamer S10-5 have been successfully obtained and characterized. These two aptamers exhibited the Kd value of 17.08 nM and 34.14 nM. AuNP-based aptasensor using these two DNA aptamers showed high specificity and selectivity for EPEC K.1.1 with LOD value of 10^5 CFU/mL, depending on the length of time incubation and also primer binding site sequence. Moreover, DNA aptamer S8-7 truncation only slightly improved the performance of AuNP-based aptasensor in detecting EPEC.K1.1. Further study is necessary to improve AuNP-aptasensor performance such as through mutagenesis approach of targeted DNA aptamers before AuNP-based aptasensor can be applied as a biosensor in Point of Care (POC) diagnosis.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-022-07786-3.

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Declarations

Conflict of interest The authors declare the competing material interests of EPEC K.1.1 as a target strain for DNA aptamer was provided by Prof. Dr. dr. Sri Budiarti from IPB University.

Research involving human and animal participants This article does not contain any studies with human or animal subjects performed by any of the authors.

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