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High Efficiency Binding Aptamers for a Wide Range of Bacterial Sepsis Agents S

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Sepsis is a major health problem worldwide, with an extremely high rate of morbidity and mortality, partly due to delayed diagnosis during early disease. Currently, sepsis diagnosis requires bacterial culturing of blood samples over several days, whereas PCR-based molecular diagnosis methods are faster but lack sensitivity. The use of biosensors containing nucleic acid aptamers that bind targets with high affinity and specificity could accelerate sepsis diagnosis. Previously, we used the systematic evolution of ligands by exponential enrichment technique to develop the aptamers Antibac1 and Antibac2, targeting the ubiquitous bacterial peptidoglycan. Here, we show that these aptamers bind to four gram-positive and seven gram-negative bacterial sepsis agents with high binding efficiency. Thus, these aptamers could be used in combination as biological recognition elements in the development of biosensors that are an alternative to rapid bacteria detection, since they could provide culture and amplification-free tests for rapid clinical sepsis diagnosis.

Keywords: Anti-peptidoglycan aptamers, bacterial sepsis agents, qPCR assays, biosensor

Introduction

Sepsis is a systemic inflammatory response that results from the presence of microorganisms or their toxins in the bloodstream, and is fatal if not treated promptly. Over 18 million people worldwide are affected annually, with a mortality rate of 40% [1]. The disease is most commonly caused by bacteria, but can also be caused by fungi, and rare cases are caused by viruses or protozoan parasites [2].

Sepsis is diagnosed by detecting pathogens in patient blood samples. The rapid microorganism detection in septic patients and close monitoring of antimicrobial resistance patterns have a direct impact on patient survival and care and the cost of hospitalization [2–4]. Faster and more accurate sepsis diagnosis could prevent the use of ineffective antimicrobial therapies, allowing clinicians to make informed decisions on antibiotic therapy earlier during disease progression. Moreover, improved diagnosis could help reduce the emergence of drug-resistant pathogens and adverse drug reaction development [4].

The current method for sepsis diagnosis involves bacterial culturing of blood samples, coupled with susceptibility testing for drug resistance, which typically yields results in 5 days. In contrast, recently developed amplification-based molecular diagnosis methods based on the polymerase chain reaction (PCR) have the potential to reduce the diagnosis time to hours [5]; however, these assays are often not sensitive enough to detect the low-level blood infection commonly found in adults [5, 6]. Thus, the requirement for microorganism enrichment and sample cleaning steps - for

which robust methods are still to emerge - means that PCR has yet to fulfill its potential to greatly accelerate and improve sepsis diagnosis.

Biosensors are analytical devices that combine a biological recognition element - often antibodies or nucleic acid (aptamer) probes [7] - with a physical transducer, to generate a measurable signal proportional to the concentration of analytes [8]. Biosensor technology represents an interesting alternative for rapid bacterial detection in samples from septic patients, because it could provide culture- and amplification-free bacterial detection, with improved or equivalent sensitivity, specificity, and reproducibility, compared with culture-based tests.

Nucleic acid aptamers are specific nucleic acid sequences that can bind with high affinity and specificity to a wide range of non-nucleic acid targets, from small organic molecules to complex proteins or whole cells [9]. Aptamers are produced by systematic evolution of ligands by exponential enrichment (SELEX), and have lower production costs and no batch-to-batch variation compared with antibodies produced in vivo [9]. SELEX methodologies follow a basic pattern: a large pool of random sequences is subjected to iterative steps of selection and amplification, resulting in a pool of molecules enriched for those with high affinity and specificity to the target, and which are then cloned and independently evaluated for their binding properties [9, 10].

In a previous work, we described the SELEX-based development of two aptamers, named Antibac1 and Antibac2, that recognize the main bacterial outer cell wall peptidoglycan [11]. Peptidoglycan is a glycan chain lattice connected by multiple peptide crosslinks, and is the only cell wall polymer common to both gram-positive and gram-negative bacteria [12]. The peptidoglycan macromolecular net coats the entire cell, and consists of two components, *N*-acetylglucosamine and *N*-acetylmuramic acid, that alternate to form the carbohydrate polymer (the glycan chain).

As expected from its target specificity, Antibac1 and Antibac2 presented a high affinity binding to the important bacterial pathogens *Staphylococcus aureus* and *Escherichia coli* [11]. In the present work, we have extended the evaluation of these aptamers by assessing their relative binding to a wide range of causative agents of bacterial-borne sepsis. We show that these aptamers bind with high efficiency to four gram-positive and seven gram-negative bacterial species. Our results suggest that these aptamers have great potential as generic probes for bacterial detection, and could be used as biological recognition elements in biosensors for sepsis diagnosis in the clinical setting.

Materials and Methods

Bacterial Strains and Culture

The following bacterial strains were used in this study: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 27853, *Proteus mirabilis* ATCC 00557, *Pseudomonas aeruginosa* ATCC 700603, *Staphylococcus aureus* ATCC 29213, *Streptococcus pneumoniae* ATCC *23855*, and *Enterococcus faecium* ATCC 29212, obtained from the American Type Culture Collection; and *Proteus vulgaris, Morganella morganii, Citrobacter freundii*, and *Acinetobacter baumannii*, obtained from the bacterial collection of the Instituto Adolfo Lutz- IAL (Brazil). Bacterial cells were cultured at 37ºC in a nutrient broth medium to an OD_{600} of 0.3 (equivalent to 10^8 cells/ml), prior to use in experiments.

Aptamers and Control Oligonucleotides

The following oligonucleotides were used in this study: 55-mer, single-stranded DNA (ssDNA) aptamers Antibac1 (5′-TCGCGC GAGTCGTCTGGGGACAGGGAGTGCGCTGCTCCCCCCGCATCG TCCTCCC-3′) and Antibac2 (5′-TCGCGCGAGTCGTCTGGGGGA CTAGAGGACTTGTGCGGCCCCGCATCGTCCTCCC-3′), and the random sequence oligonucleotide 5′-TCGCGCGAGTCGTCTGTT CCAACATAGTGTCTGATTTTCTTAATGGTAGGCGAGCCCGCA TCGTCCTCCC-3′, where internal target-specific or random sequences were flanked by the binding sites to the primers AP forward (5′-TCGCGCGAGTCGTCTG-3′) and AP reverse (5′-GGG AGGACGATGCGG-3′. Prior to use in PCRs, samples were spiked with the exogenous control nucleotide AT1, for normalization purposes for qPCR. The 183 mer nucleotide AT1 (5′-CCACTC ATGTGAGAGCCAATTGTGAAGAGCACAAAAGGTGATTTCAT TTCCTTTTGTGTAATTTGCATGTTTGAACAGACACTGTATCT GTATTGTTACAATGGATATTGATTTGGTGTTTGCAGGATCCT GGACAGAAGCAAAGGCAAAGGTATAAAAGATTTGATCCC ATTAGTGTCCAAC-3′) could be amplified using the primers 5′-CTCATGTGAGAGCCAATTGTGAAG-3′ (AT1 forward) and 5′-GGACACTAATGGGATCAAATCTTTTATACC-3′ (AT1 reverse). All nucleotides were synthesized by Integrated DNA Technologies (USA).

Aptamer Binding Assays

For the aptamer binding assay we used the method as previously described by Marton *et al.* [13]. Bacterial cells were washed twice with phosphate-buffered saline (PBS) (137 mM $NaCl_2$, 2.7 mM KCl, 4.3 mM $Na_2HPO{\cdot}7H_2O$, 1.5 mM KH_2PO_4), and then diluted to 10^6 cells/ml of selection buffer (PBS containing 1.4 mM $MgCl_2$ and 0.05% BSA). Then, cells were incubated with 200 nM of aptamers or random sequence oligonucleotides for 45 min, under agitation and at room temperature. Bacterial cell-aptamer complexes were washed three times by adding 1 ml of selection buffer and inverting the tube three times, followed by centrifugation at 8,000 ×*g* for 6 min. The supernatants were discarded and the final pellet containing the cell-aptamer complexes was resuspended in 25 µl of sterile water. Before elution, 16 fmol of the AT1 oligonucleotide was added to each reaction and the aptamers

Fig. 1. Schematic representation of the aptamers binding assay.
Bacterial cells were incubated directly with aptamers (Antibac 1 and 2) or with a random sequence nucleotide, and the mixture was incubated for 45 min at room temperature. After washing, all samples were spiked with an exogenous normalization control (AT1), and then aptamers bound to bacterial cells were eluted by heating and quantified by real-time quantitative PCR (qPCR).

were eluted by heating for 5 min at 95°C. Then, the samples were centrifuged for 5 min at 8,000 ×*g*, and the supernatant was recovered and used for ssDNA relative quantification by qPCR. A pool of random sequences was used as a reference, to evaluate relative binding. This pool is flanked by the same AP primer-binding regions found at the 5′ and 3′ ends of Antibac1 and Antibac2 (random sequence nucleotide). A schematic representation of this process is illustrated in Fig. 1.

Real-Time qPCR

Relative quantification of eluted aptamers after the binding assays was performed by SYBR Green-based real-time qPCR, in an Applied Biosystems 7500 system (Applied Biosystems, EUA), using the $2^{-\Delta\Delta C_q}$ comparative quantification cycle method as previously described by Livak and Schmittgen [14]. The following PCR conditions were used in all reactions: 95°C for 1 min, followed by 40 cycles of 95°C for 45 sec, 60°C for 20 sec, and 72°C for 30 sec, with a final extension step at 72°C for 5 min. Samples were amplified in triplicates, and reaction mixtures contained 300 mM of either the AP forward and reverse primers for aptamer or random sequence nucleotide amplification, or the AT1 forward and reverse primers for amplification of the AT1 ssDNA. Statistical comparisons were performed using the *t*-test, and considering a p-value < 0.05 as statistically significant.

Constant Dissociation K_d

K_d values for each aptamer were estimated by qPCR using non-linear regression Linewaver-Burk analysis, using the formula $1/[\text{complex}] = K_d/[C_{max}] \times 1/[\text{aptamer}] + 1/[C_{max}]$, where K_d is the steady-state dissociation constant, [Complex] is the concentration of the bacteria-aptamer complex, $[C_{max}]$ 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.

Results

Initially, we validated the qPCR method by analyzing the Antibac1, Antibac2, AT1, and random sequence amplification efficiencies. The DNA dilution versus ΔCq values plot shows that the different nucleotide amplification efficiencies were approximately equal (Fig. S1). These values were close and all nucleotides were amplified with similar efficiencies; thus, we verified that all nucleotide probes could be used

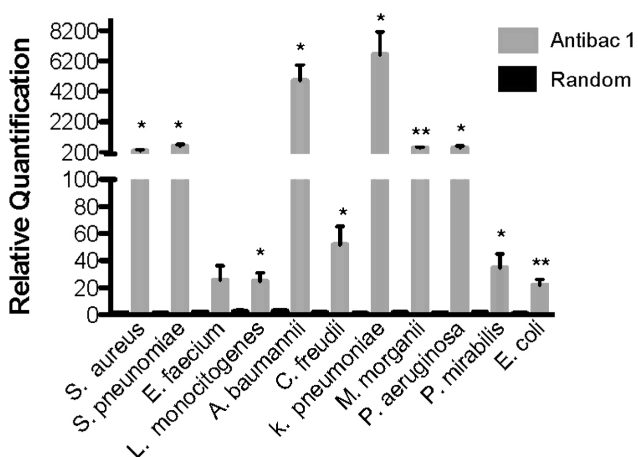

Fig. 2. Quantification of Antibac1 binding to gram-positive and gram-negative bacterial sepsis agents.
Antibac1 and the random sequence nucleotide were incubated with bacterial cells, and bound nucleotides were eluted and subjected to relative quantification by qPCR. Plots show the amount of bound Antibac1 relative to that of the random sequence nucleotide. Data represent the mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.005$.

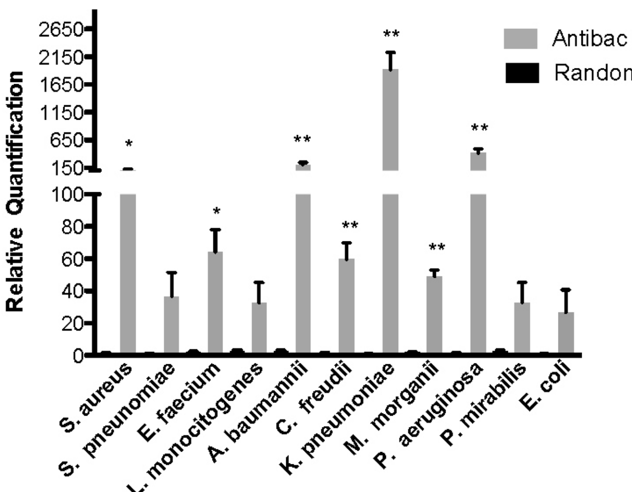

Fig. 3. Quantification of Antibac2 binding to gram-positive and gram-negative bacterial sepsis agents.

Antibac 2 and a random sequence nucleotide were incubated with bacterial cells, and bound nucleotides were eluted and subjected to relative quantification by qPCR. Plots show the amount of bound Antibac2 relative to that of the random sequence nucleotide. Data represent the mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.005$.

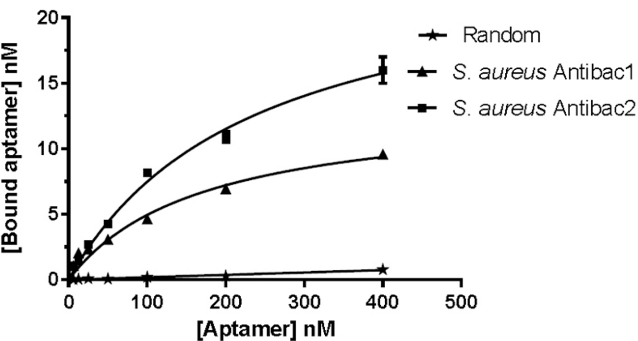

Fig. 4. Binding affinity of Antibac1 and Antibac2 aptamers to *S. aureus* ATCC 29213.

Aptamer binding was quantified by qPCR. Data represent the mean ± SD values of three independent experiments. The binding analysis was performed using GraphPad Prism 6, under the non-linear fit model for specific binding.

for relative quantification (by ΔΔCq calculation) of aptamer binding to bacterial cells.

The binding efficiency assay showed the aptamer Antibac1 bound with higher efficiency than the random sequence nucleotide to both gram-positive (*S. aureus, S. penumoniae, L. monocytogenes)* and gram-negative (*A. baumannii, C. freudii, K. pneumoniae, P. aeruginosa, P. mirabilis)* bacterial sepsis agents, with $p < 0.05$ (Fig. 2) and *M. moraganii* and *E. coli* with $p < 0.005$ (Fig. 2). Antibac2 bound with higher efficiency than the random sequence nucleotide to the gram-positive species *S. aureus* and *E. faecium* ($p < 0.05$) and to the gram-negative species (*A. baumannii, C. freudii, K. pneumoniae, M. moraganii, P. aeruginosa, P. mirabilis,* and *E. coli*; $p < 0.005$) (Fig. 3).

To assess the aptamer binding efficiency in more detail, we tested Antibac1 and Antibac2 against some gram-positive bacteria (*S. aureus* and *L. monocytogenes*) and gram negative (*A. baumanii*, *K pneumoniae*, and *E. coli*) bacterial sepsis agents by qPCR. Fig. 4 shows the binding affinity of Antibac1 and Antibac2 aptamers to *S. aureus* curve example. Both aptamers showed saturable binding to live bacterial cells, whereas the random oligonucleotide showed low or no detectable binding to *S. aureus*. The affinity constant (K_d) values at which the aptamers bind to the different bacteria are shown in the Table 1. Among the tested gram-positive bacteria (*S. aureus* and *L. monocytogenes*), *S. aureus* presented for both aptamers closer K_d values, 170.1 and 194.9 nM, and *L. monocytogenes* showed a higher binding affinity with K_d 51.74 and 54.19 nM, respectively.

Discussion

In the diagnostics field, aptamers are promising nucleic acid (ssDNA) ligands for specific recognition and binding of a variety of targets, including whole living cells [8, 15]. To date, aptasensors have been used for the detection of specific types of bacterial diseases [8], but no aptamers have been developed with the correct specificity to detect a

Table 1. Affinity constant (K_d) for Antibac1 and Antibac2 aptamers binding to several sepsis agents.

Strains	K_d [nM]		
	Antibac1	Antibac2	Random
A. baumannii	268.50 ± 54.34	71.92 ± 9.74	$1.1 \times 10^{14} \pm 3.8 \times 10^{25}$
L. monocytogenes	51.74 ± 11.75	54.19 ± 12.09	306.90 ± 66.74
E. coli	31.82 ± 4.38	62.43 ± 11.97	3.5×10^{16}
S. aureus	170.10 ± 32.13	194.90 ± 38.55	$6 \times 10^{18} \pm 3 \times 10^{33}$
K. pneumoniae	256.10 ± 47.89	195.90 ± 42.91	389 ± 151.50

large variety of bacterial sepsis agents.

Recently, we described the development of the aptamers Antibac1 and Antibac2 against bacterial cell wall peptidoglycan and demonstrated that these aptamers recognize their target molecule in the cell wall of *S. aureus* and *E. coli* [11]. In this work, we used real-time quantitative PCR to assess the Antibac1 and Antibac2 binding affinities to the main agents of bacterial sepsis, including four gram-positive and seven gram-negative bacterial strains.

The strategy used was to incubate aptamers Antibac1, Antibac2 or a random sequence oligonucleotide with bacterial suspensions, remove unbound nucleotides by washing, and then elute bound ssDNA (by heating) and perform relative oligonucleotide quantification by qPCR. The binding efficiencies were estimated by qPCR analyses. For normalization purposes, all samples were spiked with an exogenous control nucleotide (AT1).

The highest relative quantification levels were found by *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* for both aptamers. *Klebsiella pneumoniae* is a member of the *Klebsiella* genus of Enterobacteriaceae and belongs to the normal flora of the human mouth and intestine. Of the pathogenic *Klebsiella* spp., *K. pneumoniae* is the most prevalent and clinically important. Infections with *K. pneumoniae* are usually hospital-acquired and occur primarily in patients with impaired host defenses. *Klebsiella* spp. are the most commonly implicated pathogen in neonatal sepsis outbreaks [16−18]. In many hospitals, *Pseudomonas aeruginosa* has become the most common gram-negative bacterial species associated with serious hospital-acquired infections, particularly within intensive care units [19, 20]. Multidrug-resistant *Acinetobacter baumannii* is among the most prevalent bacterial pathogens associated with trauma-related wound and bloodstream infections. Septic shock and disseminated intravascular coagulation have been reported following fulminant *A. baumannii* sepsis [21, 22].

The wide range of binding affinities to bacterial strains displayed by Antibac1 and Antibac2 probably reflects differences in the cell wall peptidoglycan found in these strains. Peptidoglycan is common to both gram-positive and gram-negative bacteria [12], but variations in length, composition, and linkage of peptide side chains produce more than 100 different peptidoglycan types [23]. In many bacterial species, glycan strands also become modified, usually by *N*-deacetylation, *N*-glycosylation, and *O*-acetylation, after their insertion into the cell wall [23]. In addition, many pathogenic species contain secondary glycan strand modifications that affect their interaction with elements of the immune system [24].

The results showed that Antibac1 and Antibac2 have strong binding efficiency to different bacterial species. Although both aptamers were constructed against peptidoglycan present in greater quantity and more exposed on the outer cell on gram-positive species, the aptamers used in this study showed ability to bind to other gram-negative bacteria (*A. baumannii*, *E. coli*, and *K. pneumoniae*), and the dissociation constant value of these species was lower than that observed for *E. coli*. Whereas Antibac1 binding to *A. baumanni* showed a high K_d Antibac 2 showed to have a higher affinity. We observed a higher affinity binding using whole bacteria (nM levels) compared with the results obtained using peptidoglycan protein as target (μM levels), for Antibac1 and Antibac2, respectively [11].

The gram-negative bacterial envelope contains two distinct lipid bilayers that are separated by a peptidoglycan-containing periplasmic space [25, 26]. It was speculated that the membranes are not continuous [27] and this could explain how the aptamers can contact the peptidoglycan in gram-negative bacteria. Additionally, there are several studies showing that aptamers can be internalized in living cells for aptamer functional studies [28] and for targeted intracellular delivery [29, 30], thus indicating that the outer membrane is not a barrier for these small ssDNA sequences.

In diagnostic and detection assays, the use of aptamers offers many advantages [8]. Aptamer nucleotides are smaller, chemically stable, and cost effective, have increased resistance to pH and temperature changes, and offer remarkable flexibility in their structural design. Consequently, biosensor platforms based on aptamers, termed aptasensors, have the potential to overcome some of the drawbacks (including the loss of functional stability during storage) of antibody-based platform biosensor [31].

Several aptasensors have been developed for microorganism detection, after selection using purified targets or whole cells [15, 30]. However, most aptamers are species-specific, lacking the broad specificity to detect the variety of bacterial agents of sepsis [8]. Here, we present two aptamers that bind a wide range of bacteria sepsis agents with a high affinity level.

In this work, we showed that ssDNA aptamers targeting bacterial peptidoglycan recognize different gram-positive and gram-negative agents of sepsis, and could be used in the development of generic biosensor probes for rapid and sensitive multiplex detection of bacterial sepsis in the clinical setting, to accelerate treatment and improve patient survival. In view of the variable affinities displayed by the aptamers Antibac1 and Antibac2 for the different bacterial agents of sepsis, the combined use of both aptamers is

recommended for biosensor devices intended to help in sepsis diagnosis.

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