Selection of aptamers for use as radiopharmaceuticals in bacterial infection diagnosis

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ABSTRACT

The difficulty in early detection of specific foci in the bacterial infection caused by bacteria has raised the need to search for new techniques for this purpose, since these foci require prolonged treatment with antibiotics and in some cases even drainage or, if applicable, removal of prostheses or grafts. Detection of bacterial infections by scintigraphy has the advantage that an image of the whole body could be obtained. This study aims to obtain aptamers specific bacteria for future use as radiopharmaceutical. The SELEX (Systematic Evolution of Ligands by Exponential Enrichment) methodology can generate oligonucleotides (aptamers) that are able to bind with high affinity and specificity to a specific target, from small molecules to complex proteins, by using rounds of enrichment and amplification. Aptamers can be labeled with different radionucleotides such as $^{99m}$Tc, $^{18}$F and $^{32}$P. In this study aptamers anti-peptidoglycan, the main component of the outer cell wall of bacteria, were obtained through SELEX. The SELEX started with a pool of ssDNA that had $10^{15}$ different sequences (library), each oligo has two fixed regions merging a portion of 25 random nucleotides. Initially, the library of ssDNA was incubated with peptidoglycan, for 1h at 37ºC with stirring. Subsequently, amplification of oligonucleotides that were able to bind to peptidoglycan was performed by PCR (Polymerase Chain Reaction). The amplified oligonucleotides were again incubated with peptidoglycan, amplified and purified. At the end of 15 rounds of selection the oligonucleotides were cloned using TOPO plasmid and Escherichia coli strain Top10F’. The plasmid DNA from 40 colonies were extracted and quantified. The plasmids were sequenced using the sequencing MegaBase, and two different aptamers sequences were obtained from all clones. The aptamers obtained were synthesized and subsequently labeled with $^{32}$P in the 5’ end. The labeled aptamers were incubated with $10^7$ Staphylococcus aureus cells at 37ºC for 1 h and after centrifugation and removal of supernatant; the amount of radiolabeled ssDNA in each sample was monitored by liquid scintillation spectrometry. For one aptamer the radiation was 28 times higher than obtained with the control ($^{32}$P labeled library) and for another the radiation was 22 higher. Thus, we conclude that both aptamers showed high binding affinity for S. aureus cells.
1. INTRODUCTION

The difficulty in early detection of specific foci caused by bacteria in the bacterial infection has raised the need to search for new techniques for this purpose, since these foci require prolonged treatment with antibiotics and in some cases even drainage or, if applicable, removal of prostheses or grafts. Insufficiently eradicated infectious foci result in relapse of infection after cessation of antibiotic treatment in 12%–16% of patients (Jensen et al., 2002; Verhagen et al., 2003) and mortality rates are significantly higher in patients with metastatic complications or relapse (Lautenschlager et al., 1993). Detection of bacterial infections by scintigraphy would have the advantage that a whole body image could be obtained, since specific tracers were available. A variety of radiopharmaceuticals are used to detect infection, but long-term clinical use has shown that these probes cannot distinguish between infection and inflammation mainly because they are not specific to target bacteria, fungi or viruses (Ferro-Flores et al., 2012). Infection specific radiopharmaceuticals can be used for diagnosis, decision-making in therapy and follow-up treatments (Welling, 2009) and their development has been considered to be a very worthwhile aim for scientific research.

Aiming to develop radiopharmaceuticals for identification of infections caused by bacteria, we use the technique SELEX ("Systematic Evolution of Ligands by Exponential Enrichment") for in vitro selection of nucleic acid ligands (aptamers) capable of specifically binding to these microorganisms.

The SELEX screening is a technique that involves progressive selection of aptamers by repeating rounds of binding, partitioning and amplification from a library of random nucleic acids (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Aptamers presents similar affinity and specificity than antibodies showing a variety of advantages over them for diagnosis applications. They are minimally immunogenic, inexpensive, simple to chemically modify, easy to synthesize, have a small size which allows better tissue penetration and possess increased temperature stability over antibodies. Aptamers have a wide variety of targets ranging from small organic molecules to complex protein or even whole intact cells, they can be labeled with different radioisotopes and are emerging as a new class of molecules for radiopharmaceuticals development (Hong et al., 2011; Missailidis and Perkins, 2007). Studies using aptamers for inflammation, thrombus and tumor imaging have been published (Franciscis, 2012). The aim of this work was developing aptamers specific for bacteria identification for use as radiopharmaceuticals in the bacterial infection diagnosis by scintilography. The peptidoglycan, one of the main components of the bacterial outer cell wall, was used as target for the SELEX procedure.
2. MATERIAL AND METHODS

2.1 Microorganism strains
The Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) was maintained on BHI medium (Himedia Laboratories Pvt. Ltd.) at 37°C. The Gram-negative bacteria, *Escherichia coli* TOP10F', were grown in LB medium at 37°C. The microorganisms were harvested while in log phase growth.

2.2 SELEX library
The HPLC-purified DNA library contained two primers sites necessary for PCR amplification and a central randomized sequence of 25 nucleotides (5’ T CGC GCG AGT CGT CTG (24 random bases) CCG CAT CGT CCT CCC 3’). The library was synthesized by Integrated DNA Technologies.

2.3 Aptamer Selection by SELEX
First, the ssDNA was denatured (5nmol) by heating at 95°C for 5 min, cooled on ice at 4°C, incubated to room temperature over 1 h. The library denatured (4μL) was incubated with 20 μL of peptidoglycan (30 μg) at 37°C for 1 h with stirring, in PBS 1X. After incubation, this sample was filtered using Amicon® Ultra-0.5 (Millipore) for 10 min at 14,000 g, to segregate the binding to the peptidoglycan of nonbinding aptamers. The filter was washed three times with PBS 1X and centrifuged at 14,000 g for 10 min. The inverted filter was transferred to a new collection tube at a 1,000 g for 3 min and 10 μL of filtrate was obtained and 5 μL of this solution was used in the PCR reaction. The remainder of this sample was stored at -20°C. The concentration of target peptidoglycan used was 30 μg in cycle 1 to 4, 15 μg in cycle 5 to 8, 7.5 μg in cycle 9 to 12 and 3 μg in cycle 13 to 15.

2.4 PCR
For the PCR amplification step, 5 μL of sample to be amplified were added to 45 μL of the PCR solution containing: PCR buffer 10X, 1.5 mM MgCl₂, 0.25 mM dNTPs, 5U Taq DNA polymerase (Fermentas) and 0.4 mM of each primer (forward and reverse). The amplification was carried out for 15 cycles, each consisting of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds; followed by 72°C for 4 min. The PCR products were resolved by electrophoresis in 2% agarose gel stained with ethidium bromide and visualized under ultraviolet light system. The specific primers forward (5’-TCGCGCGAGTCGTCTG-3’) and reverse (5’-GGGAGGACGATGC-3’) with or without biotin in 5’ end were used in the PCR for the synthesis of double-stranded DNA molecules.

2.5 Purification
The obtained dsDNA sequences were then separated into single stranded form using the Streptavidin Magnetic Beads (Biolabs) following the manufacturer’s protocol. A 125 μL suspension of the beads was washed three times with binding buffer (0.5 M NaCl, 20 mM Tris-HCl and 1 mM EDTA pH= 7.5). The cleaned beads were then re-suspended in 100 μL of dsDNA and allowed to react at room temperature for 10 min
under gentle hand shaking to ensure all dsDNA was properly bound to the magnetic beads. The beads were then washed two times with 100 μL of binding buffer. The beads were resuspended in 100 μL low salt buffer (0.15 M NaCl, 20 mM Tris-HCl and 1 mM EDTA, pH 7.5) and once more in 50 μL elution buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). After that were left more 2 min at room temperature to release the desired ssDNA from its complement that remained bound to the magnetic beads, which were removed from the solution. After each round of ssDNA purification a new process of selection was carried out starting by the incubation with peptidoglycan.

2.6 Cloning
After the 14th cycle was used non-labeled primer and PCR was performed with the following program: initial heating 95°C for 2 min, followed by 15 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 min. The PCR products were cloned into pCR 4 TOPO by using TOPO TA Cloning® Kit (Invitrogen). High efficiency, *Escherichia coli* TOP10F’ cells were transformed using the vectors containing DNA fragments of interest.

2.7 Extraction of plasmid DNA
After 16h, this medium was split into two vials, one with 1.5 mL and the other with 750 μL. The latter flask were added 750 μL more of liquid LB medium with glycerol (50% v/v) and the tube was stored at -80°C. In the extraction of plasmid DNA was used Invisorb Kit® Spin Plasmid Mini Two (250), according to manufacturer’s instructions. Briefly, the tubes were centrifuged inoculum resulting from the reaction described in the cloning 14,000 g for 1 min and the supernatant was removed. The resulting precipitate was resuspended in 250 μL of solution A and stirred. Them was added 250 μL of solution B, the tubes were closed and blended by gentle inversion 5 times. After it was added 250 μL of solution C and the tubes were shaken from 4 to 6 times gently. Then the tubes were centrifuged for 5 min at 14,000 g. Subsequently, the supernatant was transferred to the filter was incubated for 1 min, centrifuged for 1 min 10,000 g and the filtrate was discarded. Were then added 750 μL of wash solution, centrifuged for 1 min 10,000 g and the filtrate was discarded. The tubes were centrifuged again for 3 min at 14,000 g for complete removal of residual ethanol. Subsequently, the filter was placed in a new tube and was added 60 μL of elution solution directly in the center of the surface of the filter was incubated for 1 min at room temperature and centrifuged at 10,000 g for 1 min for elution of plasmid DNA. Finally, after the extraction of plasmid DNA electrophoresis was performed on 2% agarose gel to confirm the extraction of oligonucleotides.

2.8 Sequencing
After plasmid extraction the DNA was measured to obtain a 250 ng sample. Then, two tubes were prepared for each sample, one with forward primer and one with reverse primer. In a tube DNase free, were added 10 pmol of forward primer, 250 ng of sample and water DNase free sufficient to complete 6 μL. In another tube was placed 10 pmol of reverse primer, 250 ng of sample and water DNase free to complete 6 μL. These samples were submitted for sequencing MegaBase.
2.9 Prediction of secondary structure
The secondary structure of the selected aptamers was analyzed using mfold program, by access for the electronic address http://www.bioinfo.rpi.edu/applications/mfold (Zuker, 2003).

2.10 Labeling with $^{32}$P
The aptamers were labeled with $^{32}$P by using T4 Polinucleotide Kinase kit (Invitrogen). Aptamers were denatured by heating at 95°C for 5 min, cooled on ice, then incubated at 4°C for 15 min and then for 1 h at room temperature. The following reagents were added to a DNase free eppendorf tube: 15.5 μL of DNase free water, 5.0 μL of reaction buffer 10X, 0.5 μL T4 Polynucleotide kinase, and 10 pmol of aptamers and 3 μL (30 mCi) of [$\gamma$-$^{32}$P] ATP (3000 Ci/mmol). The solution was heated at 37°C for 10 min. The enzyme was inactivated by heating at 65°C for 10 min to terminate the reaction.

2.10 Binding assay to Staphylococcus aureus
The binding assays were carried out by incubating in PBS 1X the radiolabeled aptamer with $10^7$ cells of S. aureus for 45 min at 37°C with shaking. Following incubation cells were washed three times with PBS 1X. After a final centrifugation for 10 min at 14,000 g the amount of radiolabeled aptamers linked to the cell pellet in each sample was monitored by scintillation counting in a Tri-Carb 2900TR Liquid Scintillation Analyser (Perkin-Elmer, Waltham, MA). The radiolabeled library was used as a control for nonspecific binding in each experiment. After resuspending the pellet in 200 μL PBS 1X was added to 1.3 mL of scintillation liquid (Ultima Gold™ LLT) for reading in the spectrophotometer Quantulus.

3. RESULTS AND DISCUSSION

Fifteen SELEX cycles were performed. The results of these cycles were shown in Figure 1. Amplification was confirmed in all cycles, since in everyone can be verified the presence of the specific band. After the separation step, up to 3 amplifications were used starting from the product of the previous amplification, to obtain enough material to begin the next cycle.
Figure 1- Cycles of SELEX. Electrophoresis in agarose gel (2%) stained with ethidium bromide. (A) 1st cycle, (B) 2nd cycle, (C) 3rd cycle, (E) 4th cycle, (F) 6th cycle, (G) 7th cycle, (H) 8th cycle, (I) 9th cycle, (J) 10th cycle, (K) 11th cycle, (L) 12th cycle, (M) 13th cycle, (N) 14th cycle, (O) 15th cycle. (1) positive control, (2) molecular weight standard 50 pb, (3) first amplifying, (4) second amplifying, (5) third amplifying, (6) negative control.

The enriched DNA pool of the fifteenth SELEX round was cloned into E. coli Top10F’ using the Kit TA-Topo vector (Invitrogen). Forty colonies were then picked out at random for sequencing of about 60 clones. Fourteen sequencing were readable and two different sequences termed Antibac1 and Antibac2 were obtained. The Antibac1 emerged from 12 clones and Antibac2 from 2 clones. The secondary structure of these aptamers sequences were analyzed by the software mfold and showed in the Figure 2.
Both aptamers were labeled with $^{32}\text{P}$ at the 5' terminus and a binding assay was performed using *S. aureus* cells. The $^{32}\text{P}$ labeled DNA randomized library was used as control. Both aptamers demonstrated high binding affinity for *S. aureus*, since radioactivity found in the cells for Antibac1 was 28 times higher than the control and for Antibac2 this ratio was 22 times higher (Figure 3).

Aptamers have been previously selected against purified bacterial components, like bacterial toxins (Vivekananda and Kiel, 2006), purified antigens (Dwarakanath* et al.*, 2004), outer membrane proteins (Bruno *et al.*, 2010) and whole cells, including *Mycobacterium tuberculosis* (Chen *et al.*, 2007), *Staphylococcus aureus* (Cao *et al.*, 2007).
2009), Streptococcus pyogenes (Hamula et al., 2011) and Salmonella typhimurium (Dwivedi et al., 2013). Aptamers against autoclaved anthrax spores were also reported (Bruno and Kiel, 1999). The main purpose of these aptamers was identifying a particular specie or strain. However, of our knowledge, this is the first study that uses peptidoglycan as a target to obtain aptamers that can be used as universal probes for bacterial infection.

Some changes must yet be performed in the Antibac 1 and Antibac 2 aptamers, to increase their stability in the blood and to allow radiolabeling with $^{99m}$Tc or $^{18}$F, before imaging studies in vivo can be performed. This report provides the first-step in the development of radiopharmaceuticals based on aptamers for bacterial infection diagnosis, offering a possible viable alternative to current methods in use.

4. CONCLUSIONS

We obtained two aptamers with high binding affinity for S. aureus. In the next step the aptamers will be labeled with $^{99m}$Tc to test its performance for in vivo identification of S. aureus infection by scintigraphy in animal model.

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