Research Article

APTAMERS IN THE DIAGNOSIS OF RICKETTSIOSIS SENSU LATO

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ABSTRACT

Aptamers are short synthetic oligonucleotides that specifically bind to various molecular targets with high affinity and selectivity. Aptamers have found two main applications in bacteriology, diagnosis in various sensing applications and riboswitches. Rickettsiosis are diseases caused by rickettsias 'sensu lato' including spotted fever, typhus, anaplasmosis, ehrlichiosis, bartonellosis and Q fever. Traditional methodologies for the diagnosis of diseases associated with rickettsias 'sensu lato' are based on serological testing, bacterial cultures and molecular assays. However, an increasing number of novel technologies, including aptamer-based diagnostic sensors, are now on the horizon, opening up possibilities for earlier diagnosis and more sensitive assays. This perspective looks at the contribution of aptamers to rickettsias 'sensu lato' diagnosis, providing information on the 'state of the art' in this emerging field..

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INTRODUCTION

Significant advances in detection and clinical diagnosis of bacterial disease have been achieved in the last decade and have opened the path for future developments (Zourob et al 2008). However, many bacterial diseases remain misdiagnosed and, despite advances in antibiotics and the availability of curative interventions, they can often lead to serious conditions and death, with diagnosis being made only retrospectively. One such group of bacteria, where methods for accurate and early diagnosis remain necessary are the "rickettsias *sensu lato*", here referred to as proteobacterias historically included in the *Rickettsiaceae* family, before phylogenetic investigations that placed *Coxiella* and *Bartonella* genus in other taxonomic subdivisions (Brenner et al 1993), (Maurin & Raoult 1999).

Various diseases are attributed to "rickettsias *sensu lato*", including spotted fever group rickettsiosis (SFGR), typhus group (TG), typhus transmitted by cat fleas, varicelliform rickettsiosis, ehrlichiosis, bartonellosis and Q fever, transmitted by arthropods like mites, ticks, lice and fleas, and found in many parts of the world.

Rickettsiosis, especially SFGR, are often misdiagnosed as other endemic diseases, such as dengue or leptospirosis in tropical countries and this can have serious or fatal results. This is due not only to the similarity of symptoms with other common endemic diseases, but also to the nature of the diagnostic assays currently employed as gold standards in the diagnosis of these conditions.

Thus, in this perspective, we look at the current diagnosis of "rickettsias *sensu lato*", including, besides the genus Rickettsia, the *Ehrlichia, Anaplasma, Bartonella* and *Coxiella* genus with a focus on the potential applications of aptamers in this field.

Aptamers in bacterial diagnosis

Aptamers are a relatively novel modality that permits recognition of almost any analyte of interest, as they are not based on selection *in vivo*, like antibodies, though they can have similar affinities and specificities to those of antibodies, but much higher than those observed by other reagents selected by combinatorial methods, such as peptides.

Very few aptamers have been described to date as antibacterial agents, such as those capable of binding to, identifying and/or neutralizing *Bacillus anthrax* (Vivekananda & Kiel 2003), a highly pathogenic bacterium that is relatively simple to produce and distribute in the field, but hard to identify and neutralize, as methodologies for that remain scarce. DNA aptamers, capable of binding and inhibiting bacterial tubercle infection, have also been described (Xiaolian & Fan

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2008), as well as aptamers against cytolysin toxin of enterococci (Morrissey & Haeberli 2003), *Bacillus cereus* (Kim et al 2009) and *Salmonella* (Kolovskaya et al 2013). However, the prohibitive cost of aptamers in comparison to traditional antibiotics limits their applicability in this field, except where antibiotic resistance or lack of adequate antibiotics make them the preferred choice.

Nevertheless, aptamers have found a number of diagnostic applications in bacteriology, in addition to various applications of riboswitches. The diagnostic applications cover not only a number of different bacterial targets, presented in table 1, but also a number of different methodologies for bacterial sensing. Thus, aptamers have been selected and used in the diagnosis of *Salmonella, E. coli, Staphylococcus, Campylobacter, Listeria* and others (Table). Equally, they have been used in a number of different diagnostic modalities, ranging from enzyme-linked immunosorbent assay (ELISA), aptamer-linked immobilized sorbent assay (ALISA), fibre-optic biosensors, various types of fluorescence detection-based assays, chemiluminescence, electrochemical, potentiometric and impedimetric sensors (Table). Finally, aptamers have been used in a number of other applications with the potential for bacterial detection, such as microarrays, although they have not been employed for this specific purpose to date.

Table: Bacteria detected using aptamers with the relevant references

Bacteria	Reference
Salmonella enteritis	Labib et al 2012, Hyeon et al 2012
Salmonella typhimurium	Maeng et al 2012, Duan et al 2012, Joshi et al 2009
Escherichia coli	Maeng et al 2012, Aguirre et al 2012, Li et al 2011, Zelada-Guillén et al 2010, Bruno et al 2010, , Lee et al 2009, So et al 2008
Staphylococcus aureus	Maeng et al 2012, Duan et al 2012, Cao et al 2009
Bacillus anthracis	Cao et al 2009, Bruno, Kiel 1999
Bacillus anthracis spores, Bacillus thuringiensis spores, MS-2 bacteriophage, ovalbumin, botulinum neurotoxina	Cao et al 2009
Bacillus thuringiensis	Cao et al 2009, Ikanovic et al 2007
Pseudomonas aeruginosa	Wang et al 2011
Campylobacter jejuni	Bruno et al 2009
Listeria monocytogenes	Ohk et al 2010
Burkholderia pseudomallei	Gnanam et al 2008
Francisella tularensis	Vivekananda, Kiel 2006

Thus, although aptamers have not yet appeared in any assays that are considered as gold standards of laboratory practice, they have already shown powerful potential in bacterial diagnosis and, where current protocols are inadequate or retrospective, aptamers will undoubtedly fulfil their promise as reagents of high applicability.

Rickettsiosis *sensu lato* and their diagnosis in humans

Rickettsiosis "*sensu lato*" are zoonosis caused by α-proteobacteria of the subgroup 1 (*Rickettsia* and *Ehrlichia*), α-proteobacteria of the subgroup 2 (*Bartonella*) and γ-proteobacteria (*Coxiella burnetti*). Although all the above species initially belonged to the order *Rickettsiales*, family *Rickettsiaceae*, they are now reorganised and considered as individual families of bacteria. The family *Bartonellaceae*, as well as *Coxiellaceae*, were removed from the order *Rickettsiales*, which now includes two families, the *Anaplasmataceae* and *Rickettsiaceae*. However, they are often all still studied within the field of rickettsiology and for the purpose of this perspective we will consider them together, particularly due to the many common characteristics they present, both in the way they are transmitted and in many of the symptoms they cause (Brenner et al 1993, Maurin & Raoult 1999).

Members of the genus Rickettsia may be classified into four groups: (i) typhus group (TG), which includes R. *typhi* and R. *prowazekii*; (ii) spotted fever group (SFG), with more than 25 different species including R. *rickettsii*, R. *conorii*, and R. *parkeri*; (iii) ancestral Rickettsiae group, which includes R. *bellii* and R. *canadensis* and (iv) transitional group, with R. *akari*, R. *autralis* and R. *felis*. The clinical features of these rickettsiosis consist of an acute undifferentiated febrile illness, often accompanied by headache, myalgia and nausea, and a maculopapular or vesical rash may be observed a few days after the onset of illness. Rickettsiosis caused by R. *conorii*, R. *africae* and R. *parkeri* present eschar and tender lymphadenopathy. SFGR and epidemic typhus are usually associated with more severe symptoms and high mortality in the absence of specific treatment, while endemic typhus is less severe and rarely fatal (Fenollar et al 2007, Fuxelius et al 2007, Valbuena & Walker 2009).

The infection can be diagnosed in a number of different ways; each approach has its advantages and its limitations. The specific laboratory confirmation could be either detection of specific antibodies by serologic tests, of DNA by Polymerase Chain Reaction (PCR), demonstration of the organism in cell culture or histopathology associated with immunohistochemistry techniques. Rickettsial antibodies can be detected by complement fixation, latex agglutination, ELISA and immunofluorescence assay (IFA). IFA is the gold standard for serological diagnostic of rickettsiosis and the diagnostic criteria of recent infection by IFA test are either a 4-fold increase of immunoglobulin IgG or IgM titers in paired samples. The main limitations with serological diagnosis include a usually negative result in the acute phase and an inability to distinguish among various rickettsial species. PCR targeting various

rickettsial genes has been developed, usually targeting citrate synthase gene (*gltA*), gene D *sca4*, surface cell antigen 1 (*sca1*), surface cell antigen 4 (*sca4*), the 17kDa surface antigen gene, and genes for outer membrane proteins A and B, *ompA* and *ompB* (*sca5*) (Prakash et al 2012).

Ehrlichia and *Anaplasma* species cause human monocytic ehrlichiosis (HME) and human granulocytic anaplasmosis (HGA), respectively, are transmitted through the bite of an infected tick vector. Clinical presentations of HGA and HME are similar and nonspecific. Fever is followed by headache, myalgias and arthralgias. A rash may be present in 10-30% of cases of HME, but is uncommon in HGA. Laboratory confirmation can be carried out using microscopy for visualisation of bacterial inclusions, mammalian cell culture and IFA. The greatest limitation of IFA is the need of a pair of serum samples from both acute and convalescent stages of the illness. In addition, PCR is currently considered as the most sensitive tool for detecting these proteobacteria during acute infection, targeting primarily the *rrs* (16S rRNA) and groESL (heat shock operon) genes (Koebel et al 2012).

Besides *Bartonella bacilliformis*, the agent of Oroya fever and verruga peruana, *Bartonella genera* have also been recognized as important agents causing human disease, including, among others, *B. henselae*, *B. quintana* and *B. elizabethae*, that are associated with a wide spectrum of clinical manifestation, such as cat scratch disease, bacillary angiomatosis, bacillary peliosis, bacteremia, endocarditis, recurrent fever, fever of unknown origin, psychiatric disorders, lymphadenopathy, splenomegaly, osteomyelitis, encephalitis, meningitis, and neuroretinites (Breitschwerdt et al 2010).

There is not a standard diagnostic laboratory examination for infections caused by *Bartonella* spp. and several techniques must be used to avoid simultaneously false negative results. Diagnostic techniques for infections with *Bartonella* spp. include IFA, culture of the pathogen, histopathological examination of lymph nodes or tissue biopsy of tissue samples, and molecular biology techniques, especially PCR.

Coxiella burnetii is a small, obligate intracellular, gram-negative, pleomorphic bacterium and is the cause of Q fever. The main characteristic of Q fever is its clinical polymorphism, and thus diagnosis can only be made by systematic tests. Infection with *C. burnetii* in humans may be asymptomatic, or may present clinical manifestations of acute or chronic Q fever, ranging from limited febrile illness, pneumonia, hepatitis and other forms of risk, such as endocarditis, and meningoencephalitis. Q fever may be detected by serological or molecular methods as well as isolation and histopathology associated with immunohistochemistry techniques. A variety of serological techniques are available, but the IFA has become the reference technique. In recent years, several PCR-based diagnostic assays have also been developed to detect *C. burnetii* DNA in cell cultures and in clinical samples. These assays use conventional PCR, nested PCR or real-time PCR conditions with Light-Cycler, SYBR Green or TaqMan chemistry (Angelakis & Raoult 2010).

Aptamers in the detection of rickettsias *sensu lato*

So far, it has been noted that serological tests where patient antibodies are being detected in ELISA or IFA assays remain the gold standard, though these are limited both by the waiting time for antibodies to be present in the patient and by the potential cross reactivity, such as that reported between the different species of *Rickettsia* and *Bartonella*. However, aptamers can be raised, and have been raised, directly against bacteria; and they have been used in ELISA type assays, such as those described by Ferreira et al (2008), and for the detection of *Francisella tularensis* in an ALISA. There, *B. henselae* was used as negative control, to show that aptamers were specific for *F. tularensis*, but did not bind to the related *Bartonella* (Vivekananda & Kiel 2006). Such a methodology, which directly recognises antigens from, or whole bacterial cells of, *Rickettsia, Bartonella, Anaplasma, Ehrlichia* and *Coxiella*, would reduce detection time and uncertainty.

Aptamers may also be directly used in the detection of rickettsias using nuclear medicine techniques. The advantage of this technique is the differentiation between bacterial and viral infections, or inflammation due to infection with microbial pathogens and inflammation due to immune response where no microbial invasion is present, as is the case, for example, in autoimmune disorders (Wareham et al 2005). This is achieved through the radiolabelling of antibiotics or other modalities and their subsequent use as imaging agents. Aptamers have already been used as specific antibiotic agents against *Salmonella*, enterococci and anthrax, and they have been previously described in molecular imaging techniques, labelled with radionuclides (Borbas et al 2007, DaPieve et al 2009), for the diagnostic imaging of disease. Thus, they have clearly the potential for selection against rickettsias and use in nuclear medicine techniques to provide more specific information about both the presence and the fate of the bacteria in the body.

Aptamers are also clearly suited to microarray development, and they have already been used in a variety of microarray formats, such as those for the capture of biomarkers in serum to be analysed and identified by Mass Spectrometry (Ahn et al 2010). Oligonucleotide arrays have been used in bacterial detection in a number of formats and for a number of applications. In rickettsiosis, there has been use of microarrays in two different forms. First, based on the whole genome sequence of *R. prowazekii*, the Rickettsial Diseases Division of the U.S. Naval Medical Research Center constructed the first rickettsial microarray with all predicted ORFs. The

genomic compositions of virulent strain and attenuated strain were studied by co-hybridization on this DNA microarray (Naval Medical Research Center website). They have also deposited a patent for the detection and diagnosis of *R. prowazekii* infection by measuring the increased or decreased expression of specific human genes following infection, using DNA microarrays and PCR. This method permits the detection of the rickettsial infection and diagnosis of epidemic typhus earlier than other available methods (Ching, Ge 2004). Another DNA array for *R. prowazekii* has also been generated, which was the first DNA microarray for the analysis of global gene expression changes in *R. prowazekii* under stress conditions (Audia et al 2008). Second, in addition to *R. prowazekii*, other *Rickettsia* microarrays are available based on rickettsial genomic information. The Rickettsia Genome microarray from Agilent Technologies, comprising probes specific to all genes and spacers from R. prowazekii, was used by Bechah and colaborators (Bechah et al 2010) for the genomic, proteomic and transcriptomic analysis of *R. prowazekii*. The genome of *R. rickettsii* str. 'Sheila Smith' has also been provided and used for the development of a database containing 3205 oligonucleotides that represent the *R. rickettsii* 'Sheila Smith' transcriptome (OligoArrayDb). Within the same database for pre-designed oligonucleotide microarray probes, available to the research community, are included the sequences for specific strains of *R. rickettsii, R. africae, R. akari, R. bellii, R. canadensis, R. conorii, R. felis, R. massiliae, R. peacockii, R. prowazekii, and R. typhi* (OligoArrayDb). These oligonucleotide arrays are based on sequences that hybridise on specific genes of rickettsias. However, oligonucleotide aptamers could be deposited on the array, recognising specific antigens on the surface of the bacterial cells, providing a powerful diagnostic approach.

In recent years much has been accomplished in the development of aptamer-based bioanalytical assays with different detection techniques (Tombelli et al 2005). The use of aptamers in bioanalytical methods is evolving, with the aim of improving analytical performance in terms of detection limits. For example, the biochemical properties of aptamers can be exploited by combining them with nanoparticles in electrochemical and optical sensors.

Numerous biosensors have been described for bacterial identification, based on different recognition elements and detection technologies independently reviewed in Zourob et al (2008).

Though the main biological sensing materials used in biosensor development are the couple antibody/ antigen, a number of other recognition elements are currently used, including aptamers (Zourob et al 2008). Other nucleic acid sensors utilise techniques available in common molecular detection assays, such as PCR or real-time PCR and subsequent detection of amplification products, but in a lab-on-chip format with integrated microfluidic platform systems and transducer/detection methodologies (Lui et al 2009).

Currently available sensor methodologies apply to the detection of rickettsias and a number of patents on sensor development for bacterial detection include rickettsias in the list of pathogenic organisms they cover. Two such examples are available. In one, the design of electrochemical sensors including electrode systems with increased oxygen generation (Simpson et al 2012) is described, where the invention includes the development of systems and methods for electrochemical analyte detection based on increased oxygen generation, and it can be applied to a number of 'contemplated analytes', including rickettsias. In a second, similar case, a transcutaneous analyte sensor is described by Brister et al (2010), for measuring analytes in a host. In this invention, it is specified that the analyte is used in a broad sense, to include, without limitation, reaction products, naturally occurring substances, artificial substances, metabolites and/or reaction products; and it subsequently specifies an extensive, but not limiting, list of chemicals, products and microorganisms, including rickettsia, that the sensor could be applied for, thus protecting the use of such sensors in rickettsial diseases.

Additionally, there are two specific sensors for rickettsia detection under development. One is from the US Office of Scientific and Technical Information (OSTI) of the Department of Energy (DOE), by Andy Hatch, describing the development of the first ultrasensitive microanalytical diagnostic method for rickettsial pathogens (Hatch 2013). This sensor uses *in situ* polymerised porous polymer monoliths both as size exclusion elements for capturing and processing rickettsial infected cells from a sample, and as a photopatternable framework for grafting high densities of functionalised antibodies and fluorescent particles. With this, they have achieved selective capture, identification of bacterial cells with at least an order of magnitude improvement in the detection limits of currently available methodologies and reduced detection times (Hatch 2013). The second current development of rickettsial sensors is again from the US, in a programme for the development of 'Handheld Aptamer-Magnetic Bead-Quantum Dot Sensor for Rickettsiae' from the US Department of Defence (Small Business Innovation Research SBIR website). In this programme, they are aiming to use a previously developed sensor format for *Campylobacter jejuni* developed by Operational Technologies, with a rapid and ultrasensitive one-step plastic adherent DNA aptamer-magnetic bead plus aptamer-quantum dot sandwich assay to detect the bacteria in a handheld battery-operated fluorimeter sensor that can be operated under field conditions. They have proposed to develop aptamers against a mixture of isolated rickettsias and to substitute those used for the *Campylobacter* detection

on the same sensor, demonstrating the applicability of aptamer sensor development for rickettsial diseases.

CONCLUSION

Aptamers are flexible, stable and economical recognition elements that can be selected against any target of interest and integrated into a variety of technologies for bacterial detection in general and rickettsial detection in particular. They offer unique benefits compared to other targeting agents, in terms of affinity and selectivity, ease of selection and synthesis, as well as temperature stability and small size. Aptamers have been utilised extensively in the development of sensors for bacterial detection. There is an extensive list of bacteria that have been used as aptamer targets (see Table 1) and a number of diagnostic technologies where aptamers have been successfully incorporated. Aptamers can be used directly in the more established and well-accepted ELISA and immunofluorescence assays; but they also offer the possibility of direct recognition of the antigen in the blood/serum, rather than indirect identification of patient antibodies, which is the current practice. They can also be used on lab-on-chip approaches that include standard techniques but on a nanoscale sensor. Finally, aptamers have already been described for rickettsial diseases in the development of novel sensors that could provide a point-of-care diagnosis of rickettsiosis *sensu lato*, which could reduce waiting times, uncertainty of results and misdiagnosis for other endemic diseases with similar clinical profile.

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