

New approaches to overcoming the limitations of clinical assays in rapid ultrasensitive identification of bacterial pathogens

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The increased incidence of emerging and re-emerging bacterial diseases, spread of antibiotic resistance and other virulence factors by horizontal gene transfer, threat of bioterrorism and the risk of food-borne infections transmitted worldwide by global logistics all challenge the applicability of classic diagnostic tools and call for new and improved strategies of pathogen detection. There is an urgent need for new techniques capable of identifying pathogens and enabling patient management before the onset of disease. An increase in sensitivity would not compromise the assays' robustness and would ease their introduction into available clinical settings. This mini-review is focused on advanced PCR-based technologies and their derivatives for detecting a range of bacterial pathogens at the whole-cell and molecular levels, and outlines the unique benefits of aptameric molecules compared to conventionally used molecular binders, such as antibodies. The availability of new tests will lead to improvements in clinical outcomes for patients, tracking of disease outbreaks and investigation of unknown pathogens.

Keywords: diagnostics of microbial pathogens; immuno-PCR; aptamers; bacterial toxins; antibiotic resistance; immuno-aptamer PCR

1. Introduction

Scientific literature widely discusses how the current level of infectious disease management is not sufficient to prevent natural outbreaks, provide an impenetrable umbrella for the threat of bioterrorism, or cope efficiently with emerging pathogens. What is debated less, given the multitude of ways in which modern technology is growing, is the direction of the development of new diagnostic tools and the approaches needed to achieve a quantum leap in performance while maintaining robustness and accessibility in current clinical settings. Why are new detection techniques needed, and what is the benefit of using them? What issues and shortcomings in detecting pathogens should such techniques address?

In the past, development of new methods for diagnosing, preventing and treating bacterial infections significantly decreased the pathogens' negative impacts on the human population, resulting in a dramatic decrease of morbidity and mortality, the end of pandemics and an increase in quality of life. However, despite major improvements, high rates of mortality from infectious diseases persist around the world, occupying first place in developing countries and yielding only to oncological and cardiovascular diseases in states with a developed or transitional economy. Globalization, ecological problems, natural evolution of pathogenic bacteria, improper treatment strategies and the threat of bioterrorism have all resulted in the rise of what NIAID Director, Anthony Fauci, called "emerging, re-emerging (resurging) or deliberately emerging diseases" [1] although 50 years ago it seemed that the eradication of the infectious diseases was possible in the very near future [2].

Fast diagnostic and etiological deciphering of a pathogen is critical to effectively countering these new threats. The outbreak of naturally chimeric *Escherichia coli* O104:H4 strain occurred in 2011, which contained unusual factors for this moderately pathogenic *E. coli* serotype such as the deadly Shiga-like toxin type 2, and antibiotic resistance [3] mean the problem of emerging atypical bacterial infections has become immediately obvious. Classic techniques cannot provide comprehensive guidance for therapeutic and epidemiological countermeasures in the case of emerging atypical pathogens. Furthermore, as revealed by this outbreak and by a bioterrorism act in United States involving anthrax spores in 2001, the time window for preventing fatalities in such cases is very narrow [4, 5].

It is necessary to note that after about 30 years of developments in molecular microbiology, for a large number of important human pathogens, including Select Agents, bacterial culture or other phenotypical tests remain the gold standard of detection and characterization. This is true, for example, for globally recognized diseases such as tuberculosis (TB) [7] and for life-threatening conditions such as poisoning by botulinum [5] or Shiga [3] toxins. Why did molecular techniques not substitute classic assays? One major drawback of the current generation of molecular-based assays is the inferiority to phenotype-based techniques in major performance aspects, especially sensitivity [6-8]. On the other hand, a major problem in classic assays is the time needed to complete the analysis. In the case of intoxication or an outbreak of a hazardous infection, time is the critical factor in preventing fatalities and other losses. An instructive example for which a wealth of data is available is provided by comparative analysis of molecular-based and culture-based assays for diagnostics of TB. In a chronic infection such as TB, the time of detection is vitally important for just a fraction of patients, such as those with XDR-TB [9] or with HIV-TB coinfection [10]. Nevertheless,

even resistance to a single first-line antibiotic is a threatening factor in TB, and significant effort has been made in developing rapid assays capable of substituting the culture test. Still, after almost two decades of research, only a few molecular assays such as the one recently developed XPERT MTB/Rif, are used as first-line TB diagnostics tools [11]. Despite the MTB/Rif assay showing high performance in TB and drug resistance detection, its sensitivity in many instances is still inferior to that of the lengthy bacterial culture test [12-14]. The field of rapid microbiological diagnostics can be split into two major parts that have much in common, but still differ in approach and in applied methodological arsenal. These fields are: detection of whole bacterial cells and detection of various bacterial components, such as toxins, virulence factors and antibiotic resistance factors.

In the case of acute infectious diseases, particularly in hazardous toxic infections, assay time is often critical for preventing fatalities and the spread of the disease. This is especially true for toxic infections such as anthrax, botulism, and enterohemorrhagic colitis with hemolytic-uremic syndrome (HUS) [3]. Common to these infections is that the major disease factor is a bacterial biotoxin, which is a complex multisubunit protein consisting of a receptor-binding subunit responsible for recognizing target cells in the host organism, and an effector subunit, which enters the cell and disables its functions by cleaving or modifying target proteins. A detailed description of the mechanism of action of hazardous bacterial toxins is outside of the scope of this review. There are a number of excellent publications describing the mechanism of action of bacterial toxins in detail, in particular, anthrax [4, 5], botulinum [6], and Shiga-like [3] two-component systems. What is worth nothing is that the major role of bacterial toxins is to disable the host's protection system, providing a favorable environment for the pathogen's propagation. To achieve this, bacteria evolved sophisticated systems of toxin delivery inside the cell, as well as a complex structure of toxins themselves, protecting them from the host defense system. One of the components of toxin's protection is its rapid accumulation in the bloodstream and ability to enter the cell quickly, where the immune system is unable to intercept it. Botulinum neurotoxins (BoNTs) enter the host by oral ingestion and are protected by toxin-associated proteins from denaturation in an acidic environment and from protease digestion [15]. The extreme toxicity of BoNTs has evolved to achieve incapacitation of the host from a tiny fraction of toxin that survives the digestive system and enters the bloodstream [16]. After entering the bloodstream, the secretion of toxins by *Bacillus anthracis*, the causative agent of anthrax is subject to 12–24 hour lag phase. During this time little or no toxin is detected and the growth of bacteria occurs without disease symptoms, followed by a burst in toxin production [17] and quick reach of the so-called "state of no-return", when any type of treatment fails to improve the patient's condition [4, 5]. No therapy is available to reverse the intoxication once the toxin enters the target cell, and imminent development of such a therapy is unlikely despite advances in medicinal chemistry over the last two decades.

Furthermore, toxigenic bacteria and bacterial toxins remain a bioterrorism threat. Lack of adequate assay systems may result in multiple cases of infection/intoxication before the dangerous agent is detected. Massive exposure to pathogens could exhaust the capacity of critical care facilities needed to treat intoxication. Last but not least, a number of bacterial toxins have been acquired by bacteria through external vectors such as bacteriophages [18] or plasmids [19]. Vector-borne toxins can be mobilized by horizontal transfer, as demonstrated by the recent outbreak of *E. coli* O104:H4 infection in Europe in 2011 [3]. The O104:H4 serotype (named after the designation of the polysaccharide surface antigen capable of inducing protective and type-specific antibodies), normally does not contain Shiga-like toxins and causes relatively mild disease [20]. The acquisition of Shiga-like toxin type 2, which is usually found exclusively in the O157:H7 serotype, and antibiotic resistance genes converted O104:H4 into a deadly pathogen that caused 50 deaths and 908 cases of HUS complication [21]. Continuing globalization of migration, food production and uncontrolled use of antibiotics mean further outbreaks caused by new bacterial types formed via horizontal gene transfer or by phage-mediated transmission of virulence factors are highly likely.

Under the current conditions, when a specific therapy for reversing intoxication is yet to be developed and the availability of antibiotics for efficient treatment is limited due the slow rate of discovering new drug candidates and spread of multidrug resistance [22], the logical solution is to develop a new generation of diagnostic tools that are superior to currently available tests in sensitivity and speed of detection. Also, new assays should have additional important characteristics that will be discussed below.

2. Important characteristics of new assays

The primary assay parameter for extremely toxic substances is the limit of detection (LOD). The lower the toxin concentration detected, the higher the likelihood of early detection of the infection and the wider the range of targets outside a clinical setting (e.g. contaminated food, water, infected soil, animal products, etc) in which the threat can be detected. Botulinum neurotoxin type A (BoNT/A) possesses 1 ng/kg LD₅₀ or even less for humans when injected into the bloodstream [22]. The dosage for lethal intoxication orally is much higher, due to toxin inactivation in the digestive system. It has been suggested that due to toxin degradation and serum clearance, the sensitivity for diagnostic evaluation must be in the low to sub-pg/ml range [6].

In the case of systemic anthrax, the toxin concentration in a patient's blood shows an extremely rapid buildup after a relatively long (12- to 24-hour) asymptomatic lag phase of infection. After this, the toxin concentration in the blood rapidly peaks to tens or even hundreds of ng/ml and the risk of reaching the "point of no return" - after which all

treatments may fail - increases dramatically [5, 6]. Therefore, the availability of an assay with sub-picogram detection of anthrax endopeptidase (lethal factor, LF) is critically important for timely initiation of therapy. The same values of assay sensitivity apply to methods of detecting the Shiga toxin, especially its most dangerous variant, Stx2, the primary cause of HUS and lethality in Shiga-related illnesses [3].

One important issue in detecting bacterial toxins and analyzing pathogen transmission routes is the need to conduct the assay with complex biological matrices such as blood, fecal and food samples [5, 6, 22]. Components of complex matrices often interfere with assay performance, e.g. by inhibiting polymerase chain reaction (PCR) [23, 24], by sequestration and degradation of the target, or by introducing nonspecific signals leading to false-positive assay readout. The same applies to the detection of other bacterial toxins, other bacterial targets and whole bacterial cells [25]. Therefore, it may not be feasible to use the entire matrix in the assay, as the risk of an incorrect result is very high. This is why the effective diagnostic assay must contain a technique for specifically isolating the target from the complex matrix (soil, blood, food, etc.).

3. Sensitive molecular tools for pathogen detection and their development in clinical tests

Immunoassays with amplifiable signals have long been considered highly specific instruments for detecting bacterial targets in complex matrices [26]. An immunoassay is a reaction in which the target is first recognized by an antibody, which then acts a signal that can be amplified in a number of ways then the recognition act is revealed by the signal amplification. The target-antibody recognition is considered specific enough to sift out contaminating matter and provide a high signal-to-noise ratio. Immunoassays are widely used in clinical practice [27]. To achieve even better specificity, sandwich immunoassays and other assay layouts have been invented (see ref. [28] for a recent review). A major drawback of conventional immunoassays in which signal amplification is achieved by an enzymatic reaction is a relatively low sensitivity level. Typical immunoassay sensitivity is in the order of 1 ng/ml of the target. Although numerous attempts have been made to increase immunoassay performance [29], no quantum leap in sensitivity has been achieved with classic signal amplification systems including the most sensitive based on bioluminescence [30]. At the same time, assay cost and complexity could increase significantly.

There has been a plethora of sensor systems developed recently, in which immuno- or related assays are integral and form the core of the detection system. A detailed description of these systems is outside the scope of this review, but is given in a number of recent publications [30-32]. Although some of the sensor-based systems display a high sensitivity and may reach the sub-picogram LOD, sophisticated techniques of the signal amplification hamper introduction of such systems in clinics. Highly sensitive sensor assays are often based on new physico-chemical principles and findings that require expensive equipment, consumables various microfabrication systems, costly materials and highly trained personnel both for assay preparation and for conducting the analysis. The wide variety of such systems under study indicates there is still no choice of the optimal device layout and assay format and these technologies need time to mature. On the other hand, judging by the information cited below, the improvement potential of currently available immuno- and related assay formats is clearly not exhausted.

There is a need for a signal amplification system that can serve as a better alternative to the existing enzyme- or fluorescence-based ones, as the degree of signal amplification is the bottleneck limiting the sensitivity of classic immunoassays. PCR is by far the most powerful amplification system in a biological setting, where a single nucleic acid molecule can be amplified into millions of copies. It was logical that PCR would be tested as the signal amplification option for an immunoassay. In this assay type, the antibody recognizing the target molecule is linked to a DNA template selected for efficient amplification. After the target recognition and removal of nonspecific matter, the antibody-linked DNA is amplified, providing a positive target-specific signal. The corresponding immunoassay type designated as immuno-PCR (IPCR) was invented 23 years ago and displayed extremely high sensitivity at the time of invention [33, 34]. On average, IPCR appeared to be about three orders of magnitude more sensitive than conventional enzyme-linked immunosorbent assay (ELISA), which can be considered a clear quantum leap in immunoassay sensitivity [34].

At the beginning, however, the range of IPCR applications was far from broad, mainly due to problems with the high background signal. IPCR's extreme sensitivity together with shortcomings in the assay design based on classic ELISA technologies often resulted in high background signals and false-positive results. Technological advances such as the invention of real-time PCR and a wide spectrum of reagents and techniques have improved IPCR specificity and sensitivity. Detailed analysis of the methodological advances that upgraded IPCR performance is outside the scope of this review, but can be found in several recent publications [35-37]. As a result of technological advances, a number of publications have reported ultrasensitive and highly specific detection of a range of biological targets, including those of pathogenic bacteria and their components, such as toxins.

4. Immuno-PCR as the template for ultrasensitive assay development

As BoNTs are the most toxic substances known to man, development of ultrasensitive *in vitro* methods to detect them has received much attention from research groups. To date, several studies have described ultrasensitive detection of botulinum toxins by IPCR. One of the most sensitive technologies is liposome PCR (LPCR) for detecting cholera and botulinum toxins. In this assay, several tens of copies of reporter DNA are encapsulated in a liposome displaying natural oligosaccharide receptor of a bacterial toxin. The toxin, captured by a monoclonal antibody, is recognized by the liposome's receptors and the encapsulated DNA amplified by PCR. The assay LOD is 12 molecules per milliliter (0.02 fg/ml) [38]. Despite ultra-high sensitivity, preparation of liposomes can be laborious, especially in terms of consistency of the liposome content, and work with such an assay system requires a high level of personnel training, which is not always possible in a clinical setting. It has been calculated that the LOD sufficient for detecting the most poisonous toxin, BoNT, is on the sub-picogram level. With this assay sensitivity, the amount of BoNT considered as dangerous in an environmental sample or in a bloodstream can be detected [6, 22]. A simplified version of the liposome PCR based on the recognition of BoNT by its ganglioside receptor, several copies of which were directly conjugated to DNA, was recently reported to have 600 aM sensitivity to BoNT/B in a food matrix (honey) [39]. This is superior to the earlier data reporting detection of BoNT by IPCR [40]. A highly sensitive IPCR assay was described in the detection of other bacterial toxins. Owing to the antibody- or ligand-based isolation of the target from complex samples, IPCR is relatively resistant to negative sample/matrix effects. Detection of staphylococcal enterotoxin B (SEB) in various food matrices with sensitivity down to 10 pg/ml has been reported [41]. The assay sensitivity can be further improved, as the LOD for SEB determination reported in another study was 0.6 pg/ml, and for SEA, 6 pg/ml [42]. New developments improving the assay format can also result in higher detection sensitivity [43]. Recent work describes the detection of the Shiga toxin small subunit (Stx2b) with an LOD of 0.1 pg/ml [44]. Other more recent studies describe the detection of another Shiga toxin subunit, Stx2a, with an LOD of as low as 10 fg/ml, based on a specially developed monoclonal antibody pair recognizing different epitopes in the toxin subunit [45]. This investigation further underscores the importance of careful selection of assay components and conditions, and their dramatic influence on the assay performance.

At the same time, a significant limitation of the antibody-based IPCR is imposed by the antibody component itself. First, it is not possible to raise an antibody "on demand" to any biomolecule, or to a given epitope of a protein. Not all targets (particularly components of the bacterial cell wall) are diverse enough and have sufficiently high immunogenicity to yield high-affinity antibodies capable of distinguishing closely related bacterial species. It is particularly difficult to raise two or more monoclonal antibodies recognizing distinct epitopes of a target. Some targets are small and contain a single immunodominant epitope, some are poorly immunogenic, the others contain epitopes that are cross-reactive between bacterial species, which also hinders correct identification [46]. With respect to IPCR assay development, preparation of antibody-DNA conjugates still can be a bottleneck, as direct conjugation of an antibody to DNA can impair immunoglobulin's binding properties, whereas streptavidin-biotin linkage may result in the increase of nonspecific signals [47].

Further improvements of the IPCR-like assays can be achieved by replacing antibodies by certain artificial binders that are not dependent on the limitations of the immune system in their ability to recognize the target, and in their affinity to the target molecule [48]. The first attempt to design such a system utilized a phage-displayed single chain antibody (scFv) or modifications thereof [49]. An scFv can be selected to bind a target using a phage display library and *in vitro* mutagenesis to improve affinity. Phage selection does not depend on the immune system, and the resulting phage can be used as a standalone tool in the IPCR: scFv (or other high-affinity binding molecule such as an "affibody" [50]) displayed on the phage surface recognize the target, whereas a unique fragment of the phage genome is used for signal amplification by IPCR.

5. Aptamers as the antibody substitutes in the PCR-based detection assays

It has been known for more than 20 years that nucleic acids can be used to generate target-specific high-affinity ligands. Such ligands are known as DNA or RNA aptamers, and the procedure for selecting aptamers is known as the Systematic Evolution of Ligand by EXponential enrichment (SELEX) [51]. Detailed description of this technology is outside the scope of this review, but there are a number of recent comprehensive reviews describing various aspects [52-55].

Since aptamers are oligomeric nucleic acids, it is obvious that they can be amplified using conventional PCR-based and related polymerase-based amplification techniques. This suggests that the IPCR assay can be implemented using an aptamer as a single type of probe. Aptamers are synthetic molecules and their production is much cheaper than antibodies. Also, the stability of aptamers is very high compared to proteinaceous compounds, making them ideal for field-based applications, and increasing the overall robustness of aptamer-based assays. Chemical flexibility of DNA synthesis permits the introduction of a wide variety of functional groups that make assay design easier, for example, oriented attachment of the bait aptamer to a solid phase in the sandwich assay. As outlined above, aptamer binders directed to different parts of a target make the development of aptamer-only sandwich-type assays feasible. In such an assay, one aptamer can be used to capture the target from solution, whereas another one as the probe to be amplified.

There are more complex and hybrid technologies available, such as universal aptamers specific to the Fc portion of the target-specific antibody, which can obviate the need of cumbersome chemical coupling of an antibody to DNA [56]. The first work utilized sandwich-based IPCR-like assays (designated by the authors as apta-PCR), yielding the detection limit of 450 fM for the thrombin target, which was 20,000 times more sensitive than the aptamer-based sandwich ELISA. Later studies resulted in 10- to 30-fold increases in the sensitivity of aptamer-based PCR detection of proteins [56, 57].

A number of modifications of apta-PCR were recently developed, including the use of rolling circle amplification [58], caged probes [59], conformational-dependent aptamers, etc. Detailed analysis of these technologies is outside the scope of this review. Several recent publications provide excellent summaries of these technologies, underscoring great flexibility in designing aptamer-based detection techniques [55, 60].

Despite promising results, there are still no reports describing the use of aptamers as antibody substitutes in IPCR-type detection of bacterial toxins and other virulence factors. At the same time, aptamers are widely exploited as substitutes for antibody-DNA conjugates, for example, in the detection of cancer-associated antigens [55]. The plausible explanation can be twofold: first, the affinity of aptamers used in these studies was lower than the average affinity of an antibody selected for ELISA or IPCR [61], and second, the SELEX procedure is still not as routine as mAb technology, and thus few laboratories focused on developing antibacterial diagnostics tools can simultaneously run aptamer selection campaigns.

As has been discussed already, the need for robust, simple and ultrasensitive techniques suitable for field-based applications will likely result in the rapid development of aptamer-based signal amplification technologies. New technologies for selecting diagnostic aptamers with improved affinity (up to sub-nanomolar range) were developed - for example, slow off-rate modified aptamers (SOMAmers) - containing modified uridine analogs incorporating various charged and hydrophobic moieties [62]. It is likely that new selection strategies based on in-depth knowledge of dynamics of association-dissociation processes of biomolecules will yield new approaches to high-affinity aptamer selection [63]. High-throughput massively parallel sequencing (next generation sequencing; NGS) provides access to the entire diversity of selected target-specific aptamers, helping to further evolve and optimize the selected pool. These and other technologies will contribute to the development of simple, robust and ultrasensitive assays detecting pathogens and their components in the near future.

6. Advances in ultrasensitive detection of whole microbial cells

Taking into consideration that not all microorganisms secrete toxins or other virulence factors and disease markers, precise determination of microbial infection causatives remains the priority of modern clinical diagnostics. Detecting whole bacteria and viruses bears certain peculiarities and limitations compared to the detection of macromolecules. For instance, it is highly desirable that such a detection system could not only sense pathogens with high efficiency, but also serve as a probe for pathogen isolation. This feature is generally redundant for the analysis of toxins and other virulence factors produced by pathogens, but it is necessary to obtain intact bacteria for further culturing, biochemical, morphological and sequence analysis. Even partial enrichment of the target species that sifts out the majority of other bacteria and growth inhibitors from typical complex samples such as blood, food, soil, urine, etc. significantly increases the likelihood of successful isolation of a pathogen [64, 65]. As bacterial cells represent a particulate matter, they are amenable to analysis and separation using cell sorting technologies (see ref [66] for a recent review). Efficiency of common diagnostic techniques currently in clinical use, and recent advances in amplification-based assays applied to the microbial cells are discussed below.

6.1 Microbiological tests, ELISA and PCR for detecting pathogenic bacteria

Centuries of thorough microbiological studies conducted by biologists and physicians have led to the isolation of most known bacterial pathogens and accumulated much knowledge about the behavior of pathogenic bacteria and their growth peculiarities. Broad panels of selective media and microbiological growth tests serve as the gold standard for identifying pathogenic bacteria in clinical settings, and represent a powerful tool for diagnosing bacterial infections. Culture-based assays offer great support for clinicians in detecting bacterial pathogens, especially taking into consideration the need to determine the antibiotic resistance spectrum of a pathogen. Morphology and differential staining analyses remain important. Currently, a pure bacterial culture is not only the ultimate evidence of a pathogen's presence, but also an invaluable source for rapid in-depth genotyping by massively parallel sequencing [67, 68].

In terms of diagnostics, however, classic bacterial isolation and culture techniques possess important limitations: the long time needed for enrichment and selective growth of the target species (typically days, but up to several weeks for slow-growing bacteria and very complex mixtures requiring stepwise selection); frequent lack of selectivity to separate closely related species and serotypes; possibility of culture contaminations; difficulties with selective enrichment of certain poorly growing or "uncultured" bacteria [69]; risks of spread of infection and others. Taken alone, a culture test cannot provide information on the range of toxins and virulence factors produced by the bacterial cell.

As noted above, the introduction of immunological (ELISA) and nucleic-acid based amplification-enhanced tests (PCR and analogs) in clinics opened routes for new diagnostic strategies. For determining proteinaceous antigens, ELISA provides high specificity and fairly low LOD (usually up to 1 ng/ml for standard colorimetric assay readout [70, 71]). Although the LOD of ELISA for whole bacterial cells is generally applicable for clinical needs in the acute phase of disease [72], it is frequently insufficient for early pathogen detection (10^3 - 10^6 infectious particles per ml depending on assay format and the detecting antibody properties [73, 74]). Thus, in many cases, laboratory diagnostic protocols combine ELISA tests with prior enrichment of samples by cultivation in selective media. Enhanced ELISA variants using fluorogenic or luminogenic substrates for signal amplification can detect lower concentrations of bacteria [75, 76], but these assays are generally less applicable in complex biological and food samples due to their higher sensitivity to contamination and significant background values.

PCR technology, the most frequently used modern diagnostic tool for determining infectious agents, is aimed at specific detection of the pathogen DNA. Direct PCR assumes amplification of certain fragments of bacterial or viral DNA with the help of thermostable DNA polymerase, and the accumulation of reaction products that can be further visualized by electrophoresis or detected by a number of other techniques [77]. PCR is fast (it takes 1–2 hours from sample collection to the test result) and in certain cases can detect bacteria at very low concentrations (up to 10–100 bacteria per gram in fecal, urine, blood and tissue samples [78-80]). However, PCR efficiency is highly dependent on the system design, GC content of the target DNA, which varies significantly among bacterial genomes [81], and the presence of PCR inhibitors in complex matrices [82]. Most commonly, the PCR detection limit for complex biological matrices does not exceed 10^3 bacterial cells per gram of the sample [83]. To avoid the influence of PCR inhibitors and increase the likelihood of successful detection, target bacteria often need to be purified and then subjected to enrichment by limited growth [64, 65]. Taking into consideration the described drawbacks of clinical diagnostic techniques to detect live bacteria, efforts in developing next-generation assays targeting live bacteria are mostly focused on improving assay specificity and sensitivity, reducing the time necessary for analysis, and on developing techniques suitable for simultaneous tests of broad panels of samples.

6.2 IPCR-based strategies for detecting bacteria

Immuno-PCR, discussed above with respect to proteinaceous targets, has proved a valuable tool for increasing the detection sensitivity of bacterial cells.

Over the last decade efficient immuno-PCR tests were developed for detecting various pathogenic bacteria (*Salmonella typhimurium* [25], fish pathogen *Pasteurella piscicida* [84], *Leptospira borgpetersenii* serovar Hardjo [85], group A Streptococcus [86], *E.coli* O157:H7 [87], *Clostridium difficile* [88] and others). One advantage of applying the affinity-based PCR-enhanced methods to whole bacterial cells is that detection can rely on the amplification of the bacterial DNA itself rather than on the nucleic acid conjugated to the antibody probe. In view of this, the simplest experimental workflow for the IPCR-like detection of bacteria in complex matrices (so-called MIPA, magnetic IPCR [25, 85]) starts from the single-step enrichment of the target cells, due to their capture on the surface of magnetic particles coated by the target-specific antibody.

As an extra selective step, captured bacteria are lysed and bacterial DNA is detected by PCR with a pair of species-specific primers [25, 84, 87]. This approach permits the detection of pathogenic bacteria in substantially lower amounts than that achieved by common ELISA schemes and helps avoid the influence of PCR inhibitors abundant in biological matrices and clinical samples.

The antibody-enhanced PCR is significantly more sensitive than ELISA, with an LOD for *Pasteurella piscicida* in fish tissues differing by three orders of magnitude (3.4 cfu/ml over 3.4×10^4 cfu/ml, respectively) [84]. The enrichment technology followed by PCR is popular for detecting food-borne pathogens, as many of them carry no prominent factors like Shiga toxins, yet can cause a serious illness [89].

In the case of Select Agents, this methodology was successfully used for detecting *B. anthracis* with a LOD for vegetative cells and spores of 10 and 100 cfu/ml, respectively [90]. The important limitation of the enrichment-amplification approach is that it is applicable mostly to viable bacteria, whereas the presence of large amounts of bacterial debris of the same origin in the sample can interfere with the antibody binding to the live pathogen, resulting in false negative result.

In a classic IPCR assay layout, the bacterial cell is captured by the solid-phase immobilized antibody recognizing a pathogen's surface antigen (e. g. an outer membrane protein or serotype-specific polysaccharide), and then probed by an antibody-DNA conjugate with the specificity to another surface antigen. The LOD for this assay type in the detection of, for example, *Streptococcus pyogenes* was about one one-thousandth of a pathogen's cell, even in the presence of 100,000 *E.coli* cells [86].

The assay duration can be reduced if the detecting antibody is chemically linked to the PCR template [18], although the benefits of this optimization are not guaranteed and are highly dependent on the structure of the antigen binding site of a given antibody, which can be sometimes blocked or destroyed by chemical modification. Thus, careful selection of the antibody labeling strategy is important for performance of IPCR-based assays [91].

6.3 Aptamers targeting intact bacterial cells and their diagnostic applications

An important advantage of applying SELEX to bacteria is that the bacteria offer the advantage of a “self” solid phase, allowing for efficient aptamer isolation without the need to generate complex selection systems [92, 93]. Despite its simplicity, whole-cell SELEX is being continuously improved, for example, by applying high throughput techniques, such as cell sorting [94] or microfluidic separation [95]. These techniques increase the probability of isolation of rare high-affinity binders and are capable of the quick generation of pools of high-affinity aptamers that can be used for combinatorial selection.

The second advantage is that bacteria carry a considerable number of surface targets, which are either unique or distributed among few (sub)species. Furthermore, in the whole-cell selection, there is no need for prior knowledge of the target type to isolate highly specific aptamer molecules. Moreover, nonspecific aptamers can be efficiently counterselected using other bacterial species of varying degrees of relationship [96]. Selective aptameric binders were obtained for numerous pathogenic bacteria, including *Francisella tularensis* [97], *Salmonella enteritidis* [98], pathogenic species of *Campylobacter* [99], *Staphylococcus* [100], *Listeria* [101], *Streptococcus* [102], *E.coli* O157:H7 [103], *Mycobacterium tuberculosis* [104] and other bacterial pathogens. There are a number of reviews discussing the selection of aptamers to intact bacteria and their application [54, 104-106]. The possibility of effectively substituting an antibody-DNA conjugate by an aptamer in IPCR-like applications has been successfully exploited in the detection of *Salmonella* [98], *E.coli* [108], *Staphylococcus aureus* [109], etc. Moreover, aptamer-based detection of targets is possible in sandwich format, utilizing a pair of highly specific aptamers for different epitopes [110].

A wide variety of assay formats for detecting whole bacteria was developed using aptamers alone and aptamer-based hybrid technologies, including direct aptameric PCR [61], immuno-aptameric PCR [56], enzyme-linked and/or fluorescent assays [111], biosensor assays [103], biochips [112], plasmon resonance systems [113], etc with picomolar LOD exceeding that known for the best antibody molecules [114]. Detection limits for aptamer PCR-based assays are a few orders of magnitude lower than that of conventional PCR tests, [61], while the time of analysis is significantly reduced. One important field of aptamer application that is outside the scope of this review but is worth mentioning is the development of new anti-infective and anti-toxin agents for treating infectious diseases [106, 115, etc].

6.4 Bacterial sorting techniques and sorting-based multiplex detection assays

Rapid diagnostics of atypical infections (for example, an outbreak of *E. coli* O104:H4 [3]) requires high-resolution genome analysis in order to quickly pinpoint the most dangerous features of the newly emerging pathogen [67].

Cell sorting is an invaluable tool for isolating pathogenic bacteria from complex mixtures. In the past, a number of molecular tools were developed for bacterial typing; however, they were unable to cope with atypical pathogens emerging, for example, due to horizontal gene transfer [116]. They gradually became obsolete with the development of next-generation massively parallel sequencing, which is capable of interrogating a single genome of a bacterial species [117]. However, the isolation of individual bacterial species from complex matrices has proved difficult; the isolation of difficult-to-culture pathogens [69] has proved especially problematic. Modern advanced cell sorters have upgraded or pre-assembled versions tailored to work with pathogens, such as incorporated isolation cabinets, laminar flow devices, sterilization, etc. Enhanced optical and fluidic systems of new cell sorters permit efficient detection and partitioning of small particles such as bacteria. A major drawback of working with clinical samples and complex matrices is the amount of interfering matter that is either fluorescent or blocks fluorescence. Therefore, as for PCR, partial purification of bacteria is needed prior to sorting.

Aptamers and antibodies have proved themselves efficient diagnostic tools in various IPCR strategies, so would be ideally suited for isolating bacteria from complex mixtures for subsequent sorting. The availability of multiple probes specific to a single pathogen would allow multicolor detection that selects the target species while providing a means for efficient counterselecting fluorescent contaminants and other bacteria [118]. Sorted single-cell samples of pathogens can be further directly submitted to NGS for analysis [117].

Multiplexing the sorting by target and color has been actively exploited recently in a bead-based xMAP Luminex platform for detecting infectious agents and virulence factors [119, 120]. According to the bead-based multiplex principle, the antigen (still typically a protein molecule) is captured by a specific antibody immobilized on the surface of fluorescently labeled microspheres (up to 500 types in Bio-Plex 3D system), which have individual color codes or spectral addresses. The captured antigen is stained by a second antibody carrying a fluorophore with a different excitation/emission wavelength. The mixture of microspheres is further analyzed by multicolor flow cytometry. The assay works efficiently for protein antigens [121] and though there are examples of the successful employment of this technique for detecting spores, viruses and bacteria [122-125], determining whole bacterial cells in multiplex bead-based immunoassays is impeded by the size similarity of bacteria and beads as well as by background light scattering on bacterial cells.

Use of flow cytometry and cell sorting is already widely approved in clinical practice, mainly for eukaryotic cells, but emerging applications of flow cytometry in microbiology have already yielded breakthrough results, such as unexpectedly high bacteria loads in normal drinking water, which were unnoticed by classic assays [126].

The information reviewed here shows that, to develop rapid diagnostics for life-threatening emerging diseases, the necessary increase in speed and sensitivity of detecting bacterial pathogens and toxins can be achieved by further development within technical paradigms that are already in use, both in clinical assays and in detecting pathogens outside the clinical setting. Known technologies still have the potential to cope with the threats from emerging infections, bioterrorism and globalization problems. Newly developed molecular tools supplementing the available instruments help increase the performance of clinical diagnostics and environmental monitoring.

New technological platforms targeting more labor-efficient, highly reproducible, automated, sensor-based assays are still in development and need considerable time and effort to be converted to clinically applicable technologies. This will open access to even faster, more personalized and robust tools for diagnosing and monitoring infectious diseases.

Acknowledgements The support by the research grant from the Russian Science Foundation № 14-15-00630 is gratefully acknowledged.

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