Review of methods used for identification of biothreat agents in environmental protection and human health aspects

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Abstract

Modern threats of bioterrorism force the need to develop methods for rapid and accurate identification of dangerous biological agents. Currently, there are many types of methods used in this field of studies that are based on immunological or genetic techniques, or constitute a combination of both methods (immuno-genetic). There are also methods that have been developed on the basis of physical and chemical properties of the analytes. Each group of these analytical assays can be further divided into conventional methods (e.g. simple antigen-antibody reactions, classical PCR, real-time PCR), and modern technologies (e.g. microarray technology, aptamers, phosphors, etc.). Nanodiagnostics constitute another group of methods that utilize the objects at a nanoscale (below 100 nm). There are also integrated and automated diagnostic systems, which combine different methods and allow simultaneous sampling, extraction of genetic material and detection and identification of the analyte using genetic, as well as immunological techniques.

Key words

diagnostic techniques, bioterrorism, immunological methods, genetic methods

INTRODUCTION

Increasing threats of bioterrorist attacks have forced the need to develop methods for rapid detection and identification of biological agents. These types of analyses are important not only to confirm the fact of a bioterrorist act, but also to choose the most effective methods of protecting public health [1, 2, 3]. To detect and identify biological agents, a number of technologies have been developed, some of which were available before September 11, 2001, and others were introduced later [4]. Although it is claimed that many of the technologies satisfy the criteria of speed, accuracy and reliability, only a few of them can be used in the field [4].

Bioterrorism, as well as natural biological contaminations, present many challenges for diagnostic methods. Some of them are designed to detect the attack, while others may be suitable for different kinds of needs (including clinical). Diagnostic methods should provide the possibility of detection and confirmation of biological risk factors, including modified and unknown factors, directly from the sample, without false positive and false negative results. The devices for detection should be portable, easy to use and capable of detecting a number of factors simultaneously [4]. Although several diagnostic methods satisfy many of these criteria, none satisfy all of them. Diagnostic assays must be sensitive and specific, without interference from

other materials. In contrast to chemical detectors that detect chemical agents in amounts threatening to human health, biological detectors can rarely detect microorganisms directly from samples at or below the risk levels, because of their low sensitivity. Diagnostic systems based on the nucleic acid amplification methods are more sensitive than systems based on the use of antibodies [4]. The PCR method enables detection of a single microorganism in a short period of time [5, 6, 7]. However, the PCR technique requires a clean sample and does not allow to the detection of toxins and particles that do not contain nucleic acids, such as prions [4]. The PCR products cannot be stored and undergo further analysis. In addition to sensitivity, specificity of the methods is also important to minimize background signals and to eliminate false-positive results with samples that are often a complex mixture of organic and inorganic compounds. Specificity can be determined not only by interfering substances, but also by high concentrations of competing antigens or DNA. In the case of PCR, the high sensitivity can also be weak because of the possibility of DNA- contaminated amplification and obtaining false positive results. In addition to sensitivity and specificity, reproducibility is another important imposed requirement of diagnostic methods. A number of factors can affect the reproducibility of the methods, including stability and consistency of reagents and the differences in assay conditions. These differences can be reduced by standardizing the assay conditions and procedures. Diagnostic methods must provide the ability to detect different biological agents in samples. This is necessary because the investigated samples can contain toxins, bacteria, viruses, or other types of compounds. In some cases, unknown biological factors may have been deliberately altered through genetic, antigenic or

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chemical modifications, or may represent new or previously unknown variants of known microorganisms, which makes the detection of biological agents extremely difficult. Even without modification, the conventional biological agents are difficult to detect in contaminated samples. Human samples (e.g. blood and faeces), as well as powders, food, water, and even air, present challenges for diagnostic methods. Anticoagulants, leukocyte DNA and heme compounds inhibit the PCR reaction [8, 9]. Fats in ground beef and a large number of accompanying bacteria in faecal samples may interfere with immunological assays. For this reason, biological agents must be isolated or purified from the samples prior to analysis and identification, which increases the time of analysis and, importantly, makes it impossible to apply in the field. Some of the viable organisms cannot be grown rapidly (e.g. *Francisella tularensis, Mycobacterium tuberculosis*) or have specific nutritional requirements. An important factor in biodetection is the type of material, the procedure of sample storage and transport. Samples derived from the water and air must be concentrated prior detection of the target factor. Air samples must be carried out in a liquid state because the majority of diagnostic methods are adapted only to liquid samples. The efficiency of recovery of the target analyte can change and affect the detection limit. The size, number and distribution of the sample, as well as time and method of transport is also an important factor, particularly in relation to viable organisms. In some cases, confirmation of microbe viability may be important to determine whether it poses a health risk (PCR method does not provide such an answer, with the exception of mRNA) [4].

Various methods for detection and identification of pathogens have been used for many years [4]. They are based on culture and biochemical tests. Although they are valuable, they cannot be used in the field, and in the event of a bioterrorist attack they do not allow detection and identification of biological agents in real time. Many financial resources have been currently invested in the development of commercial technologies that can rapidly and accurately detect and identify biological agents, both in the field, as well as in stationary laboratory conditions [4].

Immunological methods. Immunological tests, since their emergence, have been constantly improved to provide a useful tool for the detection of infectious diseases, drugs, toxins and pollutants in the medical, pharmaceutical and food industry. In addition, immunological detection has been successfully used to detect bacterial cells, spores, viruses and toxins, considering the fact that each component, which is able to elicit an immune response, can be used as antigen. Various types of immunoassays used to detect biological agents have been described [4, 10]. Typically, they can analyze only one factor in the sample, which means that to detect more than one microorganism in a sample it is necessary to perform many simultaneous or successive tests. Elaboration of assays that would enable simultaneous detection of multiple targets is still a subject of investigation [4]. The specificity of immunoassays is limited by the quality of antibodies, and their sensitivity (detection limit of ~10^5 CFU). Thus, it is generally lower than PCR and other assays based on DNA amplification. Improving the quality of antibodies (e.g. production of antibodies from recombinant libraries) and the conditions of the applied tests may increase the sensitivity and specificity of immunoassays [4].

Many different immunoassays are currently commercially available. Some of them are enzyme-linked immunosorbent assays (ELISA) that belong to the type of classic sandwich assays [4]. These assays can be performed with various substrates and labels (fluorescent, chemiluminescent and electrochemiluminescent), as well as on many types of platforms (biosensors, flow cytometry, microarrays and lateral flow devices).

In Luminex xMAP technology the basic sandwich assay format has been used: antibodies are coated on the surface of polystyrene beads instead of a plate, and the beads are then separated by flow cytometry [11]. Since each type of bead may be coated with a different set of antibodies, it is possible to perform multiple tests simultaneously. The beads are kept in solution during the process of binding the target antigen to the specific antibody, and the spectral dual laser system is used for detection. The laser detects excitation of the donor and acceptor dye of each bound antigen, wherein the signal intensity is proportional to the amount of antigen present in the sample. xMAP technology is a part of ongoing environmental monitoring systems [12] and has been used to assess human exposure to dangerous biological agents [13].

Immunoprecipitation technique. A technique based on paramagnetic bead technology applied by Bertin Technologies in a KIM device. This small ruggedized device was developed as a field unit for on-site confirmation of the presence of bio-warfare agents in a hot-zone. The performance characteristics of this equipment are very promising, even better when compared with ELISA and immune HHA. The device is designed to work together with a stand-alone bio-aerosol detector and Coriolis air sampler. Man-operated immunodetection confirms or excludes tested bio-warfare agents. A serious disadvantage of KIM is the current 'under development' status of this instrument, and limited availability of the agent-specific tests.

The BV M-series device uses electrochemiluminescence to detect reporter molecules used in the sandwich assay. In BV assays, paramagnetic beads are used as carriers of the antibodies [14]. After binding of the antigen to the reporter antibodies labeled with BV-TAG, Ru(bpy)$_3^{2+}$, the bound antigens pass through the flow cytometer and are captured by the magnet on the electrode surface, leading to their separation from other components. The electrical potential generated by the electrode excites BV-TAG (ruthenium (II), tris-bipyridine, N-hydroxysuccimid), which then emits light that is recorded by the detector. This technology is mainly used for clinical purposes, and also to detect, e.g. *Escherichia coli O157* [15, 16, 17], *Bacillus* spores [16], *Yersinia spp.* [16], *Salmonella enterica serovar Typhimurium* [17] and toxins [18].

Bio-Detector, based on the ELISA assay tape system is a stable, portable package. Liquid samples are injected and separated, and each sample mixed during the reaction with fluorescein- and biotin-streptavidin-labeled antibodies, which bind to biotin. After binding of labeled antibody to the antigen they are filtered, and then captured by a tape coated with biotin, located in different positions to each sample. Streptavidin acts as a bridge between the biotin-labeled antigen and biotin-coated tape. Anti-fluorescein antibody solution, conjugated with the enzyme urease is filtered through the tape and binds captured antigen. In

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the next step, the tape is placed over the sensor and coated with an urea solution. If the tape has bound antigen, urease hydrolysis the urea, causing a change in pH in correlation with the amount of target antigen. The sensor processes the signal, so that the data on the presence and amount of antigen present in the sample are obtained [4].

DELFIA system (dissociation-enhanced lanthanide fluorescence immunoassay) is an example of the format based on time-resolved fluorescence. This technique is based on the labels that are the complex lanthanide compounds, which have long fluorescence decay times, allowing measurements of fluorescence without interference from background signals. Typically, the assays are performed on a standard ELISA microplates (detected antibodies are labeled with lanthanides) and the label dissociates from the antibodies following the incubation, using a low pH solution. Free molecules form a new, stable chelates of high fluorescence intensity, recorded by the system. The DELFIA system is used, e.g. for detection of *Francisella tularensis*, *Clostridium botulinum* toxins and staphylococcal enterotoxin B [10].

Lateral flow devices have been developed primarily for rapid field diagnostics, but can also be useful for clinical laboratories. These are single-use assays, based on immunochromatographics or enzymatic assays, giving visible, coloured end products that confirm or exclude the presence of the factor. The system is based on the antibodies coated on cellulose or membrane, and uses capillary flow forces for the elution of antigens labeled with colloidal gold, or coloured microparticles conjugated with antibodies in the liquid phase. Positive result is achieved by binding of the labeled antigen-antibody complex with a second, immobile antibody (usually directed against immunoglobulin G) [4]. Negative result (control) is an indicator of the accuracy of the assay and passing the sample through the field test. Although this type of assays are easy to perform and fast, they are not too sensitive and give more false positive results. However, they may be useful for rapid initial screening of samples for the presence of biological agents, although, as a matter of principle, any positive result must be confirmed by other tests, such as PCR. Lateral flow devices have been developed by many companies for such biological agents as *Bacillus anthracis*, *Francisella tularensis*, *Yersinia pestis*, *Clostridium botulinum* and several toxins, such as ricin and staphylococcal enterotoxin B [4].

Immunological techniques usually use both conventional antibodies, as well as other forms of antibodies, including mono- and bivalent antibody fragments, such as Fab' and F(ab')$_2$ and single-chain variable regions, which were tested for their sensitivity, specificity and stability [19, 20, 21, 22]. These fragments can also be modified using recombination for improving the binding kinetics of antigen-antibody fragments [21]. Phage libraries allow increasing the affinity of antibodies by selecting from the thousands of possible peptides, one that shows the highest binding efficiency to the antigen. The selected antibodies or their fragments can be subsequently synthesized chemically or produced in a large amount in a recombinant host [21]. The antibodies or their fragments, being the products of phage expression, can also be used [21]. Using this methodology, the antibodies binding bacteria, e.g. *Brucella melitensis* [23], viruses, such as vaccinia virus [24], and toxins, e.g. *Clostridium difficile* toxin B [25]

and botulinum toxin [19], have been produced. The final expression products can be isolated or remain associated with the phage [21, 23, 25].

The use of aptamers and peptide ligands constitutes an alternative to antibodies. Aptamers are small DNA or RNA ligands formed using combinatorial methods, which recognize antigen on the basis of the sequence, not a spatial structure. Aptamers are used, for example, for the detection of ricin by the biochip bead sensor [26] and cholera toxin, staphylococcal enterotoxin B and *Bacillus anthracis* spores in the electrochemiluminescence assay [27]. It was found that ribozymes and autocatalytic RNA can generate a signal after binding antigen [28] and photoaptamers, and DNA aptamers bind antigen under UV light influence [29]. In short, recombinant peptide sequences are also tested for their binding and detection ability in biosensors. As phage-produced antibodies or their fragments, they can be chemically synthesized. Reports have been published on the use of synthetic sequences specifically binding ricin [30], *Bacillus anthracis* spores and other *Bacillus* species [31, 32, 33], *Staphylococcus aureus* protein A [34] and staphylococcal enterotoxin B [35].

Biochip technology. Methods for detecting antigens using biochip technology have been described recently [36]. One of latest uses dielectrophoresis for the concentration of antigens, followed by immunoelectrophoretical detection [37, 38]. The combination of immunoelectrophoresis and dielectrophoresis enables targeting of assay components and antigens in the appropriate position, and reading of the results is performed by fluorescence microscopy. This method was applied to detect *Escherichia coli O157: H7* and *Bacillus globigii* spores [38], staphylococcal enterotoxin B and cholera toxin B [38]. In the second variant, the system uses sensors on a chip of metal oxide semiconductors for the detection of antigens on the microarray [39]. All points on the microarray can be analyzed simultaneously or separately by optical detectors. This technology has been developed to detect *Bacillus globigii* spores in the air, which was possible through the integration of a portable air sampler with on-chip ELISA [40]. A similar system has also been used in the microarray immunoassay in which oligonucleotides-labeled antibodies were introduced on the chip and bound with complementary nucleotides in the matrix [41, 42]. This system has been used to detect ricin, M13 phage and *Bacillus globigii* spores, and drugs. The lab-on-a-chip technology has also been developed for rapid detection of botulinum toxins [43].

Contemporary research on new methods of antigens detection have drawn attention to the usefulness of biosensors based on the phenomenon of surface plasmon resonance. This type of sensors detect antigens directly by measuring changes in the refractive index which occur at the time of binding antigen with the surface coated with a metal (mainly gold or silver) [44]. Commercial devices are now big, but miniature versions have been developed, suitable for the detection of, e.g. staphylococcal enterotoxin B under field conditions [45, 46]. The miniature gold-coated sensor uses peptides bound with the metal surface to target the specific antigen, and to monitor the angle of reflection at different wavelengths, leading to signal generation. It is temperature-controlled and has two channels, one of which serves as a control channel. Another miniature surface plasmon resonance device uses a polished, single optical fibre to monitor spectral changes of different

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angles of incidence [47]. Such device has been used, e.g. for direct detection of staphylococcal enterotoxin B in milk [47]. The sensitivity of the assay has been increased by signal amplification using a secondary antibody [48].

Surface plasmon resonance is a physical phenomenon, applied for the first time by Genoptics in a set of SPRi devices which served for immunodetection. This instrument may be used for continuous flow monitoring of liquefied samples (e.g. when combined with any aerosol –liquids samplers). Due to lack of any markers and reversibility of antibody-antigen reaction, SPRi allows for low running costs and performance characteristics comparable to ELISA techniques. Another advantage is the high multiplexing capability of this technology. However, it may take some more time to develop an instrument with a set of ready-to-use assays for biodefence applications.

Another method using biosensors is based on the excitation by evanescent wave of fluorophores-labeled antibodies bound to antigens on the waveguide surface [44, 49]. Several such devices have been studied for their ability to detect biological agents. One of the introduced instruments – RAPTOR, uses antibodies coated on the polystyrene fibre optic waveguides. The samples pass through the waveguides, and the antigens are bound by the antibody, following binding fluorophore-labeled antibodies on the waveguides. Fluorophore molecules, whose size ranges from 100 – 1,000 nm, are excited on the waveguide surface by the evanescent range of laser light. Next, part of the emitted energy joins to the optical fibre and is measured and quantified by the photodiode. The increase of fluorescence is proportional to the concentration of antigen. The RAPTOR biosensor, and its predecessor Analyte 2000, has been used directly to detect biological agents in a variety of environmental materials, including food [4, 50], river water containing organic compounds and microorganisms [51], powders [52] and clinical samples [53, 54, 55]. An array biosensor, and biosensor of integrated waveguide have also been developed. In the array biosensor, the antigens are bound by the antibody on a slide. Fluorescently-labeled native antibodies are added and the views recorded by a camera after excitation by a laser [56, 57]. The array biosensor has been used to detect antigens in a variety of complex materials, including toxins in body fluids, environmental samples and food [56], and bacteria in food [57]. In the integrated waveguide biosensor the glass capillary tubes are used as waveguides, so that staphylococcal enterotoxin B can be detected at a level of pg/ml [56].

Cantilever technology uses mass changes to detect antigens bound on the surface of the device. Antigenic specificity is achieved by adsorption or binding of certain types of molecules to the substrate surface, which increases the mass and changes the frequency of support. The cantilever biosensor has been used to detect *Escherichia coli O157: H7* in a suspension containing 10^6 to 10^9 cells/ml [4].

Magneto-elastic cantilever immunosensor which uses magnetic fields to induce sensor vibration has been also developed [58]. In this case, the sensor surface is coated with antibodies which specifically bind to target antigens. The binding of antigen-alkaline phosphatase-labeled antibodies causes signal amplification by increasing the total mass of the sensor. This sensor has been studied with regard to detection of the presence of *Escherichia coli O157: H7* and staphylococcal

enterotoxin B, with a sensitivity of 10^2 cells/ml and 0.5 ng/ml respectively. Another tested possibility to improve the detection of *Salmonella enterica serovar Typhimurium* uses impedance analysis of a quartz crystal oscillator, which constitutes the basis for the piezoelectric biosensor [59]. Antibodies coated on paramagnetic microspheres provide the specificity and increased reactivity of the sensor. The detection limit is reported to be 10^3 cells/ml.

The next device, which has been tested to study *Bacillus thuringiensis* spores as surrogates of *Bacillus anthracis* spores, is based on surface acoustic wave technology [60]. The use of a horizontal cut wave allows the acoustic measurements in a liquid environment, and the surface coated with monoclonal antibodies provides a specific binding of the antigen. The ability to detect inhaled *Bacillus thuringiensis* spores at, or below the threshold dose for *Bacillus anthracis* infection has been indicated.

Nowadays the up-converting phosphors technology is beginning to replace the fluorescent dyes through the use of unique, submicroscopic ceramic particles containing a rare-earth element [61, 62]. These labels absorb more than one low energy photon (IR) in order to achieve a higher energy state, emitted as phosphorescence when the crystal returns to the ground state. Since this process does not occur naturally, it is possible to eliminate background signals using such labels. Hampl *et al.* [61] described a method of binding the up-converting phosphors with antibodies, and used them both in lateral flow assays, and plate assays for the detection of human chorionic gonadotropin and for simultaneous detection of gonadotropin and albumin. Niedbala *et al.* [62] applied this technology in the sandwich assay with a side flow, reaching the sensitivity of 10^3 CFU/mL *Escherichia coli O157: H7* in an enriched medium containing 10^9 organisms/ml, grown from infected ground beef. This enriched medium served as a negative control background in the assay. The usefulness of this technology has also been studied in the assays based on nucleic acid amplification [63].

MesoScale technology, principally based on the same immunodetection phase as BioVeris, uses a dry plate instead of beads. Eletro-chemiluminescence of ruthenium complex provides greater sensitivity. Since BioVeris was merged with the Roche Corporation in 2007, it discontinued ECL for application in the defence against biothreats. MesoScale launched instruments PR 2 1500, 1800 and 1900, as well as set assays related to defence application. These ruggedized models offer full automatisation of sample processing, or aerosol sampling with its processing, as well as high-throughput capability. The methods based on the MesoScale technology are able to confirm the presence of almost a full spectrum of biowarfare agents with 'ready-to-use' kits provided by the producer (including *B. anthracis, F. tularensis, Y. pestis, C. botulinum, Escherichia coli O157, Brucella*, Orthopox, VEE and some toxins). However, the availability of this technology is limited to the USA, which is a serious disadvantage for European users.

Genetic methods. Detection methods based on nucleic acid amplification use the principal of base pair complementarity to detect and identify biological agents [4, 64, 65]. Although any biological agent which contains DNA or RNA can be detected using these methods [66], their disadvantage is

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caused by the inability to detect proteins, such as toxins and bioregulators [4]. Many assays which were developed on the basis of these molecular techniques have been recently described [67, 68, 69, 70]. Some of the modern methods use isothermal amplification of nucleic acids or enable detection of the agent directly from the sample, without the amplification step [4].

Quantitative real-time PCR (Q-PCR) combines PCR amplification with simultaneous detection of amplification products, based on changes in fluorescence intensity, proportional to the increase of the product [71, 72]. There are two variants of Q-PCR, with specific and non-specific detection. The non-specific detection uses DNA-intercalating dyes that emit fluoresce when bound to DNA (e.g. SYBR Green). During DNA amplification, the dye intercalates into the reaction product, following the analysis of the melting curve (which provide specificity), whereas during the process of DNA dissociation the fluorescence signal decreases. This variant is useful for optimization of PCR conditions and verification of the specificity of primers. It is also less expensive in comparison to specific detection. In the latter, change of fluorescence intensity occurs due to the use of fluorogenic labeled probes, containing both fluorescent dye and sometimes a quencher. The increase of fluorescence indicates the hybridization of probes to the target DNA.

Q-PCR has many applications [4]. Many companies offer a wide range of combinations of primers and probes in various configurations, including TaqMan probes (double-labeled) [73], with black hole quenchers [74], lock-type probes [4], molecular beacons [75], lux-type (fluorogenic labeled primers) [76], Hyb-probes (FRET – fluorescence resonance energy transfer probes) and scorpion-type probes [77]. Each of these types has a different mechanism of monitoring the amplification process (e.g. separation of fluorophore from the quencher). As the fluorescent dyes attached to the primers and probes may have different ranges of excitation /emission, Q-PCR can be used for detection of several factors simultaneously. These dyes include: 6-carboxyfluorescein, 6-carboxytetrametylorhodamine, Cy5, Cy3, Rox, Texas Red, rhodamine, fluorescein and Oregon Green.

Although the quenchers have the ability to quench the entire spectrum of their emission, the optimal solution is to match their maximum absorption to the maximum dye emission. As technology advances, the instruments for Q-PCR are becoming smaller, faster and more sensitive, which is important for the rapid identification of biological agents. Accurate characterization and identification of bacteria by Q-PCR is limited by the efficiency of nucleic acids isolation or cell lysis, nucleic acid degradation by nucleases, the primers and probes reactivity (i.e. specificity and quantity), as well as the variability of the polymerases, buffer and thermocycler performance. The efficiency of DNA isolation or cell/spore lysis significantly affect the sensitivity, reproducibility and accuracy of each PCR method. In addition, the presence of inhibitors such as humic acids or chelating agents may interfere with probes' and primers' targets, resulting in obtaining false negative results. Despite these limitations, the analyses based on PCR are highly specific and sensitive in relation to the selected agent (usually detect 10 – 100 cells).

Automatic real-time thermocycler can quickly analyze four samples simultaneously and has brought an interesting solution in this area of research [78]. It has been successfully used for the detection of *Bacillus anthracis* from swabs taken after the last case of bioterrorism in the USA, and *Escherichia coli* in water samples. SYBR Green dye has been used for labeling, enabling positive results to be obtained within 13 – 32 min. It has been reported that *Erwinia herbicola* nocturnal culture was detected using a TaqMan probe in approximately 7 min. at a concentration with 500 cells.

NASBA technique is based on the isothermal amplification of single-stranded RNA to identify the target organisms. In this method, the primer binds to the RNA target sequence, and reverse transcriptase produces a cDNA strand. RN-ase digests the RNA, and next a second primer binds to the cDNA, which is used by reverse transcriptase to synthesize a double-stranded cDNA. In the following step, T7 RNA polymerase is used to synthesize RNA transcripts during the amplification process. This method has been applied to detect certain viruses [79, 80, 81, 82], bacteria [83], fungi [84] and protozoa [85]. Positive results of the detection of microorganisms have been obtained in both environmental [81, 83] and clinical samples [80, 81, 84, 85], which indicates that NASBA is a sensitive, specific and rapid analytical method which can also be used to detect viable organisms using mRNA as a template.

Isothermal loop-amplification is a method of DNA amplification using the new, displaced strand [86]. In this technique, the polymerase and four primers, specifically designed to the sequence of both sense and nonsense strands of target DNA, are used. This method has been used to detect some viruses, such as West Nile virus, Newcastle disease virus, influenza A virus, acute respiratory distress syndrome virus [87, 88]; bacteria, such as *Mycobacterium avium subsp. paratuberculosis, Yersinia pseudotuberculosis, Streptococcus pneumoniae, Shigella* and enteroinvasive *Escherichia coli* [89, 90, 91, 92, 93]; and fungi, such as *Paracoccidioides brasilliensis* [94]. It is also useful in the studies of clinical samples [88, 91, 93, 95]. The technique of identifying bacteria using rRNA for *Escherichia coli* in a mixture containing *Bordetella bronchiseptica* has been described by Maruyama *et al.*[92]. After binding of bacterial rRNA by a single-stranded DNA on monolayer, the bacteria were conjugated with another single-stranded, fluorescein-labeled DNA probe. In the next step, the peroxidase-labeled antifluorescein antibodies were used to obtain an amplified signal, measured amperometrically. The sensitivity of the method was approximately 10^3 cells of *Escherichia coli*/ml. Further studies based on this approach have led to development of a highly specific electronic sensor that detects mRNA at concentrations below 1 femtomol [96].

Another approach based on PCR technique, which serves to detect biological agents, uses an advanced micro-electrokinetic system. It applies platforms of universal nucleic acid amplification test (NAAT) to identify conserved and variable sequences of all human pathogens [97].

DNA-selective fluorescent dye is a novel technology that has been used in the Prime Alert system for detection of all bacteria and spores, as well as many viruses by LATE PCR (Linear After the Expotential Polymerase Chain Reaction). This system can also detect toxins, such as ricin, botulinum toxin A and B or staphylococcal enterotoxin B, using FT-IR (Fourier-Transform Infrared Spectroscopy).

PCR technique can also be combined with other diagnostic methods, such as mass spectrometry. PLEX-ID technology allows for rapid identification of microorganisms (bacteria, viruses, fungi, certain parasites) with the possibility of genotyping. PLEX-ID system is designated rather for use in stationary laboratories. All steps related to nucleic acid isolation and PCR products amplification may be carried out manually or on automated devices. PCR reaction (multiplexed or not) results in the synthesis of a number of amplicons for each sample (depending on the general panel used), which correspond to selected regions of the genome agent (e.g. 16SrRNA region, species specific region). Subsequent electrospray ionization and time-of-flight mass spectrometry allows for very accurate determination of molecular size and weight of both strands of each product. Thanks to a very broad and comprehensive molecular database, the obtained results for each product, analyzed by comparison with the available referees base, may lead to unique identification of the sample. The entire procedure, including PCR, lasts about eight hours. There are a number of detection panels available now in this technology (respiratory virus, bio-threat, broad bacteria, broad viruses, food borne, multi-drug resistance, among others). The advantages of such technology include extremely high multiplexing capability (up to 1,000s of agents) and significant throughput. These features make PLEX-ID an excellent device in the case of analyzing samples of unknown origin. On the other hand, the initial investment price may be a serious disadvantage for wider use of PLEX-ID.

Biochip technology has also been applied for the detection of nucleic acids [4, 57, 98, 99, 100, 101, 102, 103]. One of the more investigated methods is the electric-field-driven method for immunological detection of bacterial DNA. Here, the bacteria concentrated by dielectrophoresis are lysed, and the obtained DNA is denatured at high temperature, following duplication by the displaced-strand amplification [38, 100] and analysis using the 'on-chip' electric-field-driven hybridization assay. This assay has been used, e.g. to distinguish six gene sequences of different bacteria, such as *Escherichia coli, Salmonella, Campylobacter, Staphylococcus, Chlamydia* [100].

Ali et al. described the use of another chip technology, based on the detection of antibodies, and proved its suitability for the detection of amplified DNA using capillary electrophoresis and laser-induced fluorescence [102]. The DNA of enterotoxic strain *Escherichia coli* was used in this assay, demonstrating that this method was comparable with the gel analysis of PCR in terms of identification of specific amplicons; it was faster and created the possibility of multiplexing on microarray platform.

For rapid distinction of a mixture of similar 18-nucleotide DNA fragments, 'taste chip' technology was also used, applying antibody-based biochips [102], in which microspheres coated with DNA probes were placed in micro-cavities on a silicon chip, through which the samples containing the target DNA were passed. This allowed for rapid identification of a single nucleotide mismatch, obtaining the limit detection level of approximately 10^{-13} M.

These techniques can also be used to detect bacterial cells, which was demonstrated on the example of *Escherichia coli K-12* [103]. The analysis steps using this biochip included sample binding, preparation, PCR, hybridization, and electrochemical detection. The sensitivity of the method was 10^3 cells with a very low background level.

Other genetic methods are applied for forensic and phylogenetic studies. They include analysis of variable number tandem repeats (VNTR) [104], multi-spacer sequence typing (MST), multilocus-sequence typing (MLST), pulsed field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and nucleic acid sequencing. These genotyping methods give the ability to detect new, mutated strains of these bacteria.

Other diagnostic methods. Among the known detection systems there are also devices which use a response of specific cell types to toxic or infectious agents in order to identify them. In these devices, the cells constituting the sensor generate a signal which can be measured by an electrode or an optical detector. The detector cells may originate from specific single-cells organisms or tissues, such as nerve tissue or heart cells, and may be in both a primary and settled state [4].

The search for a different, improved type of cell biosensor based on B cells, is being continued. The role of B cells is to present the surface antibodies which serve as a receptors for various pathogens. Rider et al. [105] designed B cells capable of expressing aequorin and jellyfish protein that emits radiation due to the calcium ions flow and pathogen-specific surface antibodies. Although this type of cell biosensor provides high specificity, the problem of cross-reactivity of antibodies may arise. For the detection of biological agents, the chromatophores – colourful cells found in skin of cold-blooded animals – were also applied [106]. Chromatophores are responsible for pigmentation and masking in these animals and change colour on exposure to various biologically-active substances, such as pesticides, neurotransmitters and bacterial toxins. The colour change can be observed microscopically or spectrophotometrically. A biosensor that uses fish chromatophores has been developed. It has been examined with regard to its detection capability of both chemical and bacterial toxins, obtaining, e.g. the limit detection level of *Bacillus cereus* below the EPA standard [105, 106]. Since the fish chromatophores multiply very weakly, there is no need for frequent exchange of culture medium in this method.

Biosensors based on physical and chemical properties react to the characteristic features of the target analyte. Examples of these technologies include: mass spectrometry, Raman spectrometry and intrinsic fluorescence/luminescence. These methods do not require additional biological reagents; however, they may use the affinity of probes to increase the binding capacity and specificity. Use of the spectroscopic method in various wavelength UV/Vis, which utilizes both light scattering and absorbing properties of vegetative cells and spores, has also been studied [107]. The obtained data indicate that it is possible to differentiate vegetative cells of different bacteria species and to distinguish vegetative bacteria cells from spores, while the disadvantage is the need for thorough sample preparation in order to obtain a pure suspension of the material in non-absorbing medium.

There have been also many attempts to develop a biosensor based on mass spectrometry [108, 109], in which the sample components are identified on the basis of molecular weight analysis. This type of device has been used to identify bacterial and viral proteins [110] and bacterial cells [111], and also to distinguish *Bacillus thuringiensis* and *Bacillus*

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atrophaeus spores in spray [112]. Most of these methods use a matrix-assisted laser desorption /ionization time-of-flight (MALDI-TOF). The systems are fast and do not require the use of additional reagents. Moreover, a small volume of samples is sufficient for the analysis. MALDI-TOF /mass spectrometry is theoretically capable of identifying all types of biological agents, including viruses, bacteria, fungi and spores. The disadvantage of this method is the need for a high concentration of the investigated factor (10^5–10^7 cells/ml for analysis of whole cells), development of spectral analysis databases for each factor, and the possibility of losing specificity in mixed samples. The specificity of the method can be increased by flow fractionation, involving the separation of the particles in the mixture prior to analysis by MALDI-TOF /mass spectrometry [113].

EIS-FTICR – Electrospray Ionization Fourier Transform Ion Cyclotron Resonance mass spectrometry has been used for simultaneous analysis of alleles of variable number tandem repeats, and in studies on single nucleotide polymorphism [114]. In this method, DNA is amplified and the size of the amplified fragment reduced to less than 200 bp, which is optimal for mass spectrometry. The analysis of the PCR products of the variable number tandem repeats and single nucleotide polymorphism in *Bacillus anthracis*, using EIS-FTICR-mass spectrometry gave results comparable to traditional gel electrophoresis. Fourier-Transform Infrared Spectroscopy (FT-IR) has been also used in HazMatID 360 and HazMatID Ranger portable devices which are capable of identifying over 32,000 substances, e.g. white powders.

SERS – Surface-Enhanced Raman Scattering is a technique also under investigation in regard to its use as a method of identifying pathogens' nucleic acids and toxins. Similar to mass spectrometry, this technique requires a comprehensive analysis of each factor. Two methods for detection of dipicolinic acid present in bacteria spores using SERS have been described [115, 116]. Bell et al. increased the sensitivity of detection of dipicolinic acid by the addition of sodium sulfate and thiosulfate as an internal standard, which allowed a quantitative analysis [115]. Zhang et al. [116] used a silver film on glass nanospheres to detect the dipicolinic acid isolated from *Bacillus subtilis* spores, obtaining a sensitivity of approximately 10^3 spores in 20 µl. These authors also tested the portable detector, whose sensitivity has been determined as approximately 10^4 spores in 20 µl. SERS technology has been used in the biochip platform where specific antigen-binding antibodies have been used [117]. The obtained data indicate the possibility of identification of *Listeria* species, *Legionella*, *Bacillus* species spores and *Cryptosporidium parvum* and *Cryptosporidium meleagridis* using this biochip. These authors indicated that by application of SERS analysis it is possible to distinguish viable and dead organisms, and to identify specific toxins in the mixture. The optical single-particle binding using near-infrared SERS method [118] has been studied to distinguish spores of two strains of *Bacillus stearothermophilus*. This method has been also successfully used for distinguishing seven *Escherichia coli* strains and six clinical isolates from urinary tract infections [119]. For diagnostic purposes, SERS and related surface-enhanced resonance Raman scattering (SERRS) technology were also used. In this case, the analyzed factor was directly labeled with the active substance [120], or indirectly by gold or silver nanoparticles bound with the SERS /SERRS-active components [121, 122, 123].

Another example of the use of SERS-active components was described in a study in which two oligonucleotides were detected using the new microfluidic platform, and spectra and detection limits for eight dye-labeled oligonucleotides were analyzed using SERRS [120]. Cao et al. [121] were able to distinguish six different DNA strands and two different RNA strands using a sandwich hybridization technique on the biochip platform, and a SERS-active dye-labeled nanoparticle probe, obtaining a detection sensitivity on the femtomolar level. Other authors [122] have detected rat and goat immunoglobulin G by sandwich assay, using antibody-coated colloidal gold nanoparticles. The possibility of detecting hepatitis B surface antigen using antibodies-labeled SERS probe in sandwich assay has also been reported [123]. SERS/SERRS probes may potentially increase the specificity of the Raman scattering method and eliminate the need to create a spectra database for each factor. The optical scattering phenomenon has been used in BARDOT (Bacteria Rapid Detection using Optical scattering Technology) technology which was applied for classification of bacteria belonging to *Salmonella spp.* [124].

Among other up-to-date diagnostic technologies one should also mention Interferometric Reflectance Imaging Sensor (IRIS) used for the detection of the H1N1 virus [125]; immunomagnetic biosensors [126]; microphysiometric devices based on electrokinetics, which are used for detection of cells and bacteria [127]; MBA (BioThreat Multiplex Assay) microfluidic system for the detection of *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, *Burkholderia pseudomallei*, *Burkholderia mallei* and *Variola major* [128]; as well as Si technology based on microinductive devices [129]. A combination of protein screen and pH screen technology has been used in a BioCheck Powder Screening Test Kit based on identification of all protein containing powders. This kit enables a simultaneous detection of multiple possible biological threat agents, particularly anthrax, ricin and botulinum toxin.

Diagnostic nanotechnologies. Nanobiotechnology involves the areas of knowledge exploring and controlling structures, processes and functions of biological systems at a nanoscale (objects less than 100 nm). Nanotechnology creates new possibilities of monitoring human and animal health, as it can be used in inventing a new generation of biosensors (nanobiosensors and various kinds of chips) for microbiological diagnostics [130].

Quantum dots are the inorganic fluorescent nanocrystals that have the ability to change colour by changing the size and composition of the crystal core. They have a wide spectrum of absorption and a narrow emission peak, which makes them ideal for using in multiplexing. Furthermore, they are highly photostable and have a high performance. They are used as labels instead of conventional fluorescent dyes such as fluorescein and rhodamine. They have been used, for example, in the detection of *Escherichia coli O157: H7* [131], *Cryptosporidium* and *Giardia spp* [132, 133], and in the multiplex assay for the detection of cholera toxin, ricin, Shiga toxin 1, and staphylococcal enterotoxin B [134]. In order to increase the quantum efficiency, a new method based on nanoparticles coated with silicone (SQDNPs) has been used [135].

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Several studies have described the use of nanoparticles for detection purposes. Zinc oxide nanostructures were applied in a method of *Bacillus anthracis* detection [136]; use of nanowire sensors [130, 137, 138], or biosensors with magnetic nanoparticles [139, 140] has been reported for the detection of many other biomolecules; a sensitive nanooscillator served for detection of many pathogens [141]; a rapid and sensitive nanobiodetector based on polianiline nanofibrils was shown to be useful for detection of yeast and bacterial cells [142]; biodetector of the 'ON-OFF' type has been utilized for identification of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis* [143]. Nanomaterials based on bioamplification strategy have also been used [144]. Tran et al. [145] used a multi-layered, polianiline nanowire carbon film (PANI-MWCNT) polymerized on a platinum electrode (IDA) for the detection of Human Papilloma Virus (HPV) infection.

NIDS – Nanointeligent Detection System the for rapid detection and identification of various biological agents (toxins, bacteria, spores and viruses) in various complex samples has been developed [146]. For rapid distinction between Gram-positive and Gram-negative bacteria a porous silicon nanodetector has been developed [147], in which micropores are coated with silicone antibodies against the receptor for lipid A, and bound Gram-negative bacteria show red photoluminescence. Other authors [148] studied the possibility of using bioassay based on bioconjugates of nanoparticles for quantitative analysis of pathogens *in situ*. Bioconjugates of nanoparticles provide extremely high fluorescence signal and can be easily incorporated with other molecules, such as antibodies. Nanoparticles conjugated with antibodies can specifically identify a broad spectrum of bacteria, such as *Escherichia coli O157: H7* by the interaction of antigen with antibody, which enables to obtain a sensitivity of 1 – 400 *Escherichia coli O157* cells in contaminated beef samples. These results indicate a possible widespread use of nanoparticle bioconjugates in many biodetection systems that will be developed for the purposes of biotechnology and medicine. The incorporation of bionanotechnology into the complex biological systems will be a revolutionary tool for ultrasensitive detection of virulence markers and infectious agents.

Integrated detection systems. In recent years, integrated detection systems have been developed on a wide scale. **APDS** – Autonomous Pathogen Detection System is designed to provide civilians with an early warning in the event of a terrorist attack. The final APDS will be completely automated, offering aerosol sampling, in-line sample preparation fluidics, multiplexed detection and identification immunoassays, and orthogonal, multiplexed PCR (nucleic acid) amplification and detection. APDS has been used to detect, e.g. *Bacillus globigii*, *Erwinia herbicola*, MS2 and albumin individually, or in a mixture, as well as for simultaneous detection of *Bacillus anthracis* and *Yersinia pestis* in the air [149].

Idaho Technology Inc., a company that originated from the University of Utah in the USA, launched in recent years various innovating instruments for laboratory and field analysis of biological warfare agents.

RAPID and RAZOR, molecular PCR-based analyzers, are especially well-known types of equipment in the CBRN community. Recently, Idaho Technology has presented a Film Array technology which combines self-contained automatic nucleic acid isolation and its amplification. The entire procedure is carried out in a special apparatus which is equipped with two Peltier devices, LED and digital camera for fluorometric signal excitation and measurement, as well magnetic elements for analytes separation and pneumatic pumps for their movement through punch channels. The entire reaction is processed in a small self-contained macro-fluidic film array punch, which protects against cross-contamination and significantly reduces manual operation. Ceramic beads beating method and paramagnetic beads are applied for nucleic acid release and its subsequent isolation. It may deal with and isolate both RNA and DNA, which allows the inclusion of important RNA viruses into the panel of detected agents. In the case of RNA, cDNA is synthesized via reverse transcriptase reaction. Amplification and analysis steps constitute the most interesting part of the procedure. They comprise a two step PCR, which resembles the well-known nested PCR reaction. In this case, there is a massive (with dozens of primers) first step PCR, which results in first stage products. Subsequently, there is a specific second step which takes place in one of 96 or 120 dots, where primers for specific agents are pre-spotted and freeze-dried. This physical separation of single second step reaction, reduces many disadvantages and complications related to traditional one-tube multiplex PCR assay. The second stage PCR is a type of real-time PCR assay with fluorescent dye for monitoring of amplified product number. After reaction, melting curve analysis is carried out for final confirmation of the amplified product [150].

Currently, only a panel for the detection of respiratory viruses is available (about 15 viral agents), but others are also being developed: BWA panel (17 agents, 29 molecular target), gastro-intestinal panel (25 agent including bacteria, viruses and protozoa) and blood culture ID panel (29 targets including bacteria (G+/G), fungi and antibiotic resistance genes). The advantages of this 'lab-on-chip' system, such as the multiplexing capability or increased overall sensitivity of the assay, promote this instrument to be used in field laboratory conditions or point-of-care scenarios. The only limitation of this technology is its low throughput: one sample per 60 min is not a significant result when compared with other competing detection systems [150].

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