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Editorial

Innovations in Detection of Deliberate or Accidental Contamination with Biological Agents in Environment and Foods

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In 2016, two special issues were launched and attended in this journal, "Challenges in New Technologies for Security" (http://www.mdpi.com/journal/challenges/special_issues/tech_security) and "Food Microbiology: Technologies and processes, microbiology analysis methods, and antimicrobials" (http://www.mdpi.com/journal/challenges/special_issues/food-microbiology). In this editorial, I will review one of the topics of biological hazards detection, namely, the rapid analysis of biological agents (DNA and antigens of fungi, bacteria, and viruses) and their toxins.

Biological hazards have become a mainstream concern recently, since the terrorist attacks in several EU countries, due to the potential of individual threats posed by pathogen agents.

Agents for potential biological contamination of food supply chains include organisms responsible for diseases of humans, animals and plants, from all biological groups: viruses, bacteria, fungi, protozoa and small invertebrates. Additionally, toxins produced by some of these organisms can be used as contaminants. Biological agents can be used to contaminate food products, or in an attack on crops and farms, or can be dispersed in air and water. All biological entities involved in food production or processing, as well as many contaminants, can be recognized by analyzing nucleic acids or proteins present in the product, using biotechnology-based systems. Methods for identifying contaminants require that accepted components or ingredients can be distinguished from unwanted or noxious components. Recent research activities have focused on the development and testing of innovative and reliable analytical methods for identifying biological components present in foods, such as *L. monocytogenes* [1], *Salmonella* spp. [2], *Campylobacter* spp., enterohemorragic *E. coli* [3], *S. aureus* [4,5], *Clostridium* spp., *Bacillus anthracis*, just to mention the most relevant and frequent in occasional or deliberate contamination of foods and environment.

Innovations in direct analysis of DNA or proteins detected in the environment have been made that provide recognition of the pathogen according to data stored in sequence databases. These techniques speed up the process of identifying of the pathogen, at the same time they allow real time monitoring of critical environments taking samples at fixed time-points and acting as prevention against terrorist attacks. Genetic and molecular analyses are based on biochemical reactions, usually involving arrays of molecules for in series or in parallel analyses. These can be carried out in miniaturized conditions. Microfluidic applications for miniaturization of classical analytical techniques, such as Lab-on-a-chip, were developed in fact for defense and countermeasures against terrorism.

Several constituents are integrated into a Lab-on-chip system, from sonication devices, to microfluidics, sensors for various compounds (DNA, proteins, chemicals), label-free (SPR, SERS, Raman spectroscopy) or label-based (fluorescence) detectors of antigens, toxins, membrane components (Dipicolinic acid in spores) [6] and nucleic acids [3], direct amplification of DNA (using isothermal amplification or thermal cycling DNA amplification), and e-DNA platforms.

The possibility of deliberate introduction of biological agents in the environment or in food products, as in the case of blackmailed Anthrax toxin, has led to the development of portable detection systems able to identify this threat [6–10].

The identification of unwanted biological components in the air, water, or food products can be performed through the identification and classification of the genetic material belonging to animals, plants or micro-organisms (fingerprint), used in producing or processing the food products. The search for pathogen contaminants in food conventionally involves the isolation of micro-organisms from food, their growth or enrichment in selective media and their identification with biochemical and immunological assays. The process can be long and biased by false negatives.

Early detection is the main requirement in counteracting deliberate release in environment and foods with biological agents harmful to human safety. Biotechnology-based systems can contribute to this. The time required for identification of pathogens or chemicals is a critical issue, because it determines the speed and effectiveness of product withdrawal and recall from availability to the public. In particular, it is the identification of the contaminating agents in the air, on the surfaces and in foods.

The food industry is challenged either by deliberate introduction of hazards (as in the case of acids and chlorine introduced in bottled water), or due to accidental contamination of foods during the manufacture process. One case is the USA-based company producing whole plant extracts used as flavoring agents in gravies, soups and similar types of products, that are going to be recalled back, since products may contain *Salmonella* spp. Bacteria that are still alive.

The containment level within the limits accepted by law of fungi and bacteria in food products is strictly regulated, especially for psychrotrophic bacteria, during the manufacture and the distribution of the products. There are solutions by food technologists in order to ensure that food products remain sterile (not contaminated) during the production line, from detection in the manufacturing lines to packaging materials provided with sensors or hazard detector strips. In coming years, the food safety challenge will require manufacturers to ensure product safety from the delivery of the commodities to their distribution, from stocked products to supermarket desks. The costs of analyses can be afforded by the military agencies, but it still remains impracticable for student refectories and school meals.

In the last few years, microbiology has made great advancements through next-generation sequencing (NGS) methods. Bacteria and viruses can be sequenced, provided that sequencers by Pacific Bioscience (PacBio) or Nanopore portable tools are spread in the general laboratory, making it possible to identify the hazard, and discriminate them at strain or serotype level, detecting the presence of pathogenicity genes. Bacterial communities present in fermented foods can add increased complexity to these analyses, but these bottlenecks may be circumvented by ad hoc DNA purification and amplification methods.

A highly sensitive molecular method is the analysis of the nucleic acids, either DNA or RNA, of the micro-organisms that are believed to be contaminants. The use of quantitative Real Time PCR (RT-PCR) can allow quantification of bacteria, and the unambiguous identification of the bacterial species. Sensor methods have been applied to pathogen identification, based on the following elements: an antibody-based bioreceptor (immunosensor for capturing the analyte), linked to physicochemical detector based on one of several transducing (label-free or label dependent) mechanisms, and on signal amplification, processing and display systems. When the system is aimed at recognizing a DNA sequence, prior to or after DNA amplification, it is called a genosensor.

Several methods are available for rapid detection of multiple pathogenic components at the same time. One of the most advanced is based on DNA microarrays for detection of multiple pathogens. The advantage of the microarray approach is in the simultaneous analysis of several targets in one experiment, where hybridization of target DNA to specific probes confirms the identity and therefore enables recognition of the contaminant.

One problem is the high dilution of biological and chemical compounds, and the difficulty of recovery from heterogeneous materials such as food and complex biological substances. The problems related to methods of capture and concentration of the targets by using aptamers, biotinylated sequences, molecular imprinting and antibodies, make this a bottleneck in the sample enrichment process to achieve the maximum yield.

One possible approach is the development of assays to be applied in the field. Interest in Real Time-PCR for biological agents is dependent on the deployment of in-field tests, based on portable RT-PCR systems.

Another essential step towards the implementation of field applications for early detection is the development of DNA extraction-free methods, to avoid lengthy procedures before a diagnostic PCR or immunological analysis.

Taq polymerase is the principal enzyme used for thermal cycling DNA amplification. There are several mutant enzymes that have been exploited by companies, to be adapted to different needs. Taq polymerase has a low fidelity in copying the template, with a rate of 1:100,000 bases being erroneously incorporated in the DNA copy [11]. A high-fidelity polymerase can process to copy DNA with 1 mutation in 1 million bases [12]. Therefore, it is necessary to know which level of fidelity the experiment has to accomplish, or the level of detection sensitivity. With sequencing, laboratories request polymerases to amplify thousands or hundreds of thousands of amplicons. For DNA extracted from blood, several new polymerases are going to substitute Taq for its sensitivity to heme. Finally, GC rich sequences tend to dissociate with difficulty and remain sticky; therefore, additives are exploited to lower the melting point of GC rich sequences [13].

Among the wide range of phagic and bacterial polymerases used in isothermal amplification with high processivity, are *Bst* DNA Polymerase, a strand-displacing DNA polymerase, used in LAMP (Loop mediated isothermal AMPlification) reactions, or the bacteriophage phi29 DNA polymerase used in Rolling Circle Amplification (RCA). Recently the LAMP reaction has been put forward from on-tube qualitative amplification to a quantitative instrument based reaction [14–16].

Description of Technology Gaps

The development of methods is envisaged to integrate biomaterials in functional devices with acceptable costs and production rate. A further challenge concerns the development of methods suited to use such biomaterials as building blocks to prepare architectures with innovative or enhanced properties; for a given biomaterial, the capability to produce a large number of identical units; maintaining the nano-size and organization of nanostructures, avoiding unwanted self-assembly or coarsening phenomena.

A complete integrated system linking information about history and movement of the products along the chain, and objective parameters of quality and safety measured at critical points, would certainly increase preparedness towards intentional and accidental threats.

Microfluidics are applied to different types of sensors and chips [5,17], for detection of volatile molecules, dissolved molecules, proteins, nucleic acids, and in amplification and direct detection of DNA fragments through arrays or e-DNA platforms. The peculiar behavior of nanostructures, which is not reproducible at a larger scale, provides further possibility for specific reactions.

Innovation in Lab-on-Chip Components

Biomaterials can be molded to take desired forms and shapes, and as part of a complex system, applied to control the interactions with components of complex systems.

The Design of Biomaterials, Polymer Synthesis and Characterization, Self-Assembly of Biopolymers (Nucleic Acids, Proteins, Lipoproteins or Sugar Linked Proteins, Polysaccharides, other Compounds with Specific Features)

For Biopolymers, specific sequences in nucleic acids or amino acids provide specific features (interaction and binding with target molecules) so a SELEX combinatorial approach is widely used to identify the sequences better interacting with the desired targets. Biomaterials can be synthesized in the laboratory using a variety of chemical approaches utilizing metallic components, ceramics, or nanoscale composite materials/nanocomposites. Biomaterials may possess self-assembly properties and a structural hierarchy. Functionalization of polymer surfaces can provide the desired properties

of a surface thin layer: the technique allows the control of the gating properties, like the opening and closing of channels that controls the flow of ions between two sides of a surface. Technologies for fabricating microprocessors, capable of creating features smaller than 100 nm, are applied to nanofabrication. Molecular nanotechnology is especially associated with a molecular assembler, a machine that can produce a desired structure or device adding individual units of biomaterials to form a layer on the surface. Other performances can be obtained through the exploitation of hierarchical architectures, featuring the presence of different biomaterials and/or structures organized at different levels and length scales. Stability has been addressed, and has resulted in both improving the quality of materials (for instance, by reducing the presence of defects that can induce drift with time and reduced reproducibility) and the development of different protocols (such as photo-activation of chemical reactions).

Evolutionary trends are envisaged in the development of techniques working in parallel, manipulating (orientating) biomaterials over more device substrates at the same time. Similarly, other techniques have been devoted to directly grow biomaterials over functional substrates (Self-assembly techniques); the assembly of strong, flexible biomaterials with optical and electronic abilities leading to foldable surfaces and sensors. Sensitivity and selectivity of nanofabricated biomaterials are an important requisite of these systems. "DNA origami" has been developed as a means of producing plasmonic structures that contain nanoparticles with chiral structure arranged in helices, with a tunable optical response, with nanometer precision. The optical response of these assemblies can be rationally tuned for handedness, color and intensity, highlighting the value of DNA origami as a valuable tool for guiding the self-assembly of nanoparticles into materials with desired electric or magnetic properties.

The evolution trends are in the nanotechnology and in improvements of purification processes, the application of nano-HPLC systems, use of micro-columns for enrichment of selective targets and micro-concentrators with selective membranes.

Several innovative devices based on automatic sampling and analysis of nucleic acids or proteins have been recently developed for monitoring high-risk locations, especially for defense against bioterrorism. One trend therefore concerns sampling for surveillance and treatment of samples, which must be minimized prior to analysis.

To increase the speed of analysis, lab-on-chips are the most advanced applications of portable biosensors, reproducing complex analytical techniques on small slides of a few square centimeters. Nowadays, many analytical techniques have been integrated or associated with Lab-on-chip sensors, including PCR, RT-PCR and microarrays.

Finally, data analysis and integration with other information for efficient decision support is an issue that current research is addressing.

These evolutionary trends have been clearly expressed in the latest call for research projects in the open EU calls (2016–2017) on novel kits for detection of pathogens for humans as well as for plants.

Technology Readiness Level (TRL)

Studies on biomaterials for biological hazard detection systems at an intermediate level (TRL 6): communication between needs and applicability of prototypes to focus on specific features are required. The next steps require: miniaturization of analytical devices (Lab-on-Chip, Point of Care diagnostics) and field portability and interoperability; new strategic approaches for simultaneous identification of agents; definition of sampling strategies and treatments; interconnection with ICT systems for automatic acquisition and analysis of data.

Biotechnology-based systems must be implemented for early detection with rapid environmental DNA/protein detection, DNA-extraction-free Real Time PCR methods, or by solid-state DNA extraction methods, to monitor high-risk contamination events. To increase the speed of analysis, portable biosensors (Lab-on-chips) are needed for deployment near-line in critical points. Monitoring and surveillance of critical points against intentional or accidental delivery of biological hazards

requires the development of sampling strategies and of devices for automatic sampling at an industrial level.

Conflicts of Interest: The author declares no conflict of interest.

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