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Rapid field identification of harmful microorganisms

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Sammanfattning

Det hot som olika mikroorganismer utgör mot människors hälsa och livsmedelsproduktion har de senaste åren blivit ett allt mer komplext problem. Bidragande orsaker är, förutom ökade resor och transporter av varor, även utvecklingen inom bioteknologi. Som en konsekvens av detta har alltfler tillgång till olika typer av mikroorganismer och kunskap hur dessa på olika sätt kan modifieras, t.ex. ökad antibiotikaresistens eller toxinproduktion. Traditionellt har man fokuserat på ett fåtal högriskorganismer inom den militära sektorn (*the dirty dozen*), men hotbilden är numera för komplex för att som idag nästan uteslutande satsa på utveckling av metoder för identifiering av specifika mikroorganismer. Förutom behovet av att utveckla breda och känsliga metoder som kan svara på vilka organismer som finns i ett prov, och inte bara om organism X eller Y finns i provet, finns ett behov av att kunna utföra analyserna snabbt på plats. Detta behövs för att riskbedömningar och lämpliga motåtgärder ska kunna göras så snabbt som möjligt. Denna rapport sammanfattar de tekniker för identifiering av mikroorganismer som idag är tillgängliga eller för närvarande är under utveckling och som dessutom är snabba (under 60 minuter), fältmässiga (kunna användas utanför laboratoriemiljö) och breda (kunna detektera ett stort antal organismer).

Nyckelord: B-detektion, snabbdetektion, mikroorganismer

Summary

Biological risks, compared to a few decades ago, have become more complex. Several factors are believed to have contributed to this. These include new achievements in life sciences and biotechnology, the increase and globalisation of the biotechnology industry, both of which have led to significantly more individuals having access to knowledge and microorganisms, as well as a large increase in international travel and global trade. Consequently, microorganisms are more accessible than ever and, in addition, the proliferation of technology has made it easier for non-state actors to enhance existing pathogens, e.g. antibiotic resistance, toxins, or engineer new pathogens allowing them to remain undetected by traditional detection methods. In most biodefence programmes, only high consequence agents have been targeted for preparedness, but currently they constitute just a part of all possible biothreats. From this, it can be concluded that it cannot be justified to continue developing assays only directed at single targets. Instead, we need more generic assays that can cover a larger part of the biological risk spectrum. Combined with this challenge of generic detection is the demand for rapid on-site detection. This report summarises techniques for microorganism identification that are currently available or under development, and that are also rapid (preferably within 60 min), broad (can detect many different organisms), and fieldable (can be carried and used outside laboratory environments).

Keywords: B-detection, rapid detection, microorganisms

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1 Focus and delimitation of the report

This report focuses on:

- Rapid (preferably within 60 min), broad (can detect many different organisms), and fieldable (can be carried and used outside laboratory environments) techniques.
- Identification, here defined as determining the identity of an organism down to, at least, the genus level (preferably species). Although toxins are not covered in this report, some techniques will also be able to detect toxins and this is mentioned where applicable.

but is delimited to:

- Not considering sample collection
- Not giving prices for equipment and consumables as these change very quickly and are dependent on volumes

2 General introduction

Biological risks, compared to a few decades ago, are now so complex that it has become more appropriate to refer to a biological risk spectrum (Figure 1). Several factors are believed to have contributed to this. These include new achievements in life sciences and biotechnology, the increase and globalisation of the biotechnology industry, both of which have led to significantly more individuals having access to knowledge and microorganisms, as well as a large increase in international travel and global trade. Consequently, microorganisms are more accessible than ever and, in addition, the proliferation of technology has made it easier for non-state actors to enhance existing pathogens (e.g. antibiotic resistance, toxins etc.), or engineer new pathogens allowing them to remain undetected by traditional detection methods. In most biodefence programmes, only high consequence agents have been targeted for preparedness, but currently they constitute just a part of all possible biothreats. From this, it can be concluded that it cannot be justified to continue developing assays only directed at single targets. Instead, we need more generic assays that can cover a larger part of the biological risk spectrum. Combined with this challenge of generic detection is the demand for rapid on-site detection. Bearing this in mind, this report will focus on rapid (preferably within 60 min), broad (can detect many different organisms), and fieldable (can be carried and used outside laboratory environments) techniques for the identification of microorganisms.

Figure 1. The Biological risk spectrum (Taylor 2006)

2.1 Detection at speed – concepts and requirements for biodetection instruments

Historically, biological warfare agents were primarily designed to spread through the air. Therefore, the military have had a long-standing interest in developing instruments that detect airborne microorganisms to warn and protect military personnel against a possible attack with biological substances. While different fields have adopted different interpreta-

tions of the term “biodetection instrument”, the military definition states that a biodetection instrument is one that unambiguously warns when airborne infectious microorganisms or harmful agents are present [1].

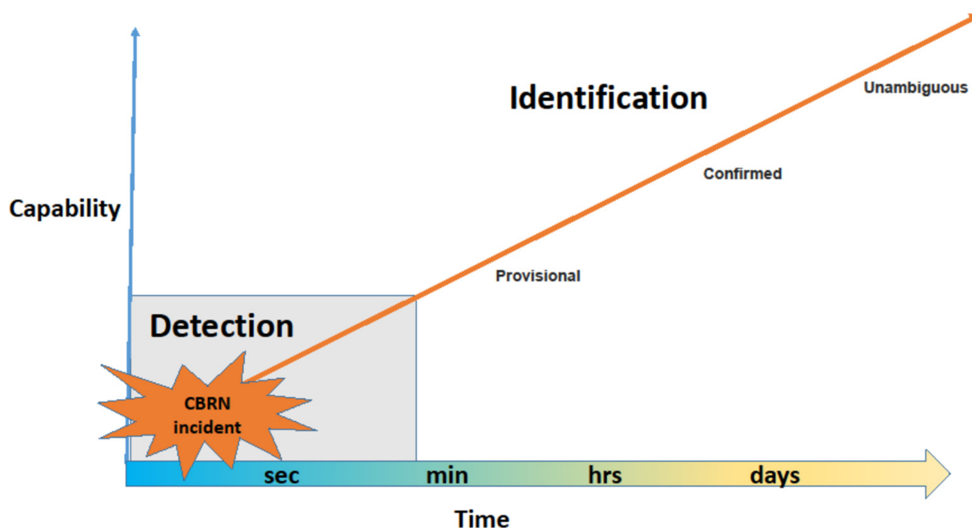

Figure 2. Detection, Identification and Monitoring, DIM, capability concept.

Both NATO and EDA have adopted the DIM concept (Figure 2), where the primary objective of detection is to discover the presence of health-threatening levels of CBRN (Chemical, Biological, Radiological and Nuclear) substances (Alerting), before they have a negative impact on mission effectiveness and to provide timely information permitting forces to adopt an appropriate level of individual and collective protection (Warning). An essential part of the DIM concept is the assumption that detection and identification represent a continuum with increasing confidence levels as one progresses towards unambiguous identification. Detection is the discovery of a hazard before it negatively impacts on the mission outcome, whereas identification is the process of characterising and determining the specific agent that was detected, to ensure the deployment of appropriate hazard management procedures and medical countermeasures. Further, the DIM concept stipulates that the quality of the results will increase with time especially when unknown hazards have to be identified. Depending on the required information level, the quality of information differs between provisional, confirmed and unambiguous identification (NATO AEP-66)[2].

In Sweden, actors in the civil sector (first responders and the police) follow, in principle, the scheme outlined in Figure 2, where the results of on-site analyses are considered provisional and a reach-back facility is necessary for interpretation of the results and/or confirmative analyses. However, the central dogma of the DIM concept i.e. that the quality of the results will increase with time, may not necessarily be true – it depends on the method used for analyses, and new methods may be developed that are both rapid and give high resolution in the same analysis run.

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[1] This definition of detection is a part of the CBRN detection, identification and monitoring (DIM) capability concept adopted by both NATO and EDA (Figure 2).For more information see: NATO. 2011. Chemical and Biological Warfare Agents (CBWA) Early Warning and Detection Triptych. AC/225(JCGCBRN)D(2011)0003 (PFP), dated 3 August 2011.

[2] NATO. 2015. NATO handbook for sampling and identification of biological, chemical, and radiological agents (SIBCRA). Ed. A., ver.1., April 2015.

Ideally, such an instrument should respond quickly, and it should also be able to respond with great certainty and sensitivity to any type of harmful microorganism. The whole system needs to be rapid, robust, and insensitive to operating temperature. In order to facilitate operation, the need for maintenance and consumables should be small. It should be a user-friendly system requiring a small degree of expert knowledge and training, as well as minimal sample preparation time prior to analysis. However, no such instrument currently exists.

The reason why there is no ideal biodetector or on-site analysis instrument for microorganisms currently on the market is because there are many biological challenges and aspects to take into consideration when analysing microorganisms in environmental samples. When analysing chemical, radioactive or nuclear materials, there is a defined particle/agent to analyse that gives a strong signature compared to the environmental background; this is not the case when analysing microorganisms in an environmental background. Biological warfare agents, as all living organisms, continuously evolve depending on the ambient environment. This means that genetically close neighbours, with which the pathogens share common ancestry and can be almost identical to, are also present in the environment. These close neighbours are, by definition, usually not pathogenic for humans or animals, or plants of economic value and, for this reason, are poorly characterised and often unknown. Thus, they may be the source of false positive reactions. Methods based on phenotypic character may also be inaccurate when applied to environmental samples, since microorganisms may change substantially and rapidly as a response to variations in ambient chemical (e.g. pH, nutrient levels etc.) and physical (temperature, osmotic pressure etc.) conditions. In addition, some pathogens have a very low infectious dose (corresponding to pictogram levels), and if the identification system does not exhibit a high level of sensitivity, this could potentially result in a false negative result. Further, to be infectious, a microorganism needs, by definition, to be alive. The microorganism can be dead or incapacitated due to e.g. single mutations and thus unable to be infectious. Thus, ideally information on the functionality or viability of the microorganism is also needed. Read more about issues of specificity/sensitivity and living/dead in info boxes 1 and 2.

Info box 1 – Sensitivity/specificity

The performance of a test is commonly measured in terms of sensitivity and specificity. A highly sensitive test reports few false negative results e.g. pathogens in samples are rarely missed. A highly specific test reports few false positive results e.g. identified pathogens in a test are rarely a false indication. False positives in the methods described in this report usually comes from closely-related non-pathogenic organisms and false negatives are common in methods that e.g. require large amounts of input material but where only very small amounts are available.

There is usually a trade-off between sensitivity and specificity, so increasing one usually means the other has to decrease. It is impossible to construct a detection method with 100% sensitivity and 100% specificity. This means that all traces of pathogens can never be detected and a negative result does not guarantee that no pathogen is present. Ideally, the sensitivity should be high enough to detect harmful amounts of any given pathogen.

False positive rate: The rate at which a target (in this context pathogenic organisms) is incorrectly determined as present.

False negative rate: The rate at which a target (pathogenic) organism is incorrectly determined as absent.

Info box 2 - Living or dead?

It might seem an easy task, but determining whether a microbe is dead or alive is complicated. The delineation between life and death is complex and debatable. In short, it is generally accepted (but not a universal rule) that in order to be considered alive, a cell must be intact, capable of reproduction and be metabolically active. Viruses, while not technically "alive" by these (and many other) definitions, can be infectious or inactive, and distinguishing between the two states can be more difficult than distinguishing living and dead forms of other microorganisms. The gold standard to assess viability is based on the ability to culture microbial cells, but many organisms need very precise conditions or enrichment, and others (the majority) will not grow at all (so-called viable but not cultivable). Thus, the distinction between dead or living microorganisms is challenging using current microbiological methods and none of the methods described in this report will determine whether microorganisms are alive or dead. However, there are methods available that address different aspect of viability, that are compatible with the focus of this report on rapid and fieldable methods. These methods typically address either the existence of an intact functional cell membrane, the presence of cellular metabolism or the presence of RNA.

For example, viability PCR (vPCR) is a method that relies on exclusion of propidium monoazide (PMA) in live cells with intact membranes and passage through damaged membranes. The PMA reacts with free DNA and blocks amplification by PCR, and thus when the sample is analysed in parallel with and without PMA, the level of viability can be assessed. Microscopy-based live/dead staining is similar to vPCR and based on membrane-impermeant DNA staining. Dead cells are quantified relative to total cells by fluorescence microscopy or flow cytometry. Further, molecular viability testing (MVT) is a method that uses the pre-rRNA synthesis in response to nutritional stimulation. The sample is divided into two aliquots: one of the aliquots is exposed to nutrients (culture medium) and, if viable cells are present in the sample, then pre-rRNA (measured by reverse transcription [RT]-qPCR) increases in the stimulated aliquot, relative to the control (non-stimulated) aliquot.

3 Current biodetectors

Many different concepts have been developed to meet the requirement of B-detection. Instruments that measure basic parameters such as size and shape of particles have been developed (e.g. BIRAL[3], ASAS technology – Aerosol size and shape). These types of instruments can distinguish, for example, spherical diesel fuel particles from bacterial spores or spore clusters. Other more advanced biodetectors utilise molecular fluorescence, which is based on the phenomenon that certain molecules absorb light at specific characteristic wavelengths. After the initial light excitation and some internal energy conversion processes, light is emitted at longer wavelengths (lower energy). Depending on the molecular structure, the fluorescence characteristics differ between compounds both in terms of intensity and wavelengths. All biological organisms have a metabolism that produce certain metabolites (e.g. NADH and riboflavins) with fluorescence properties in the UV range. The fluorescence properties of these molecules can be used for distinguishing biological particles from other particles. Each particle is illuminated and the emitted light passes through an optical filter, only transmitting fluorescence to a highly sensitive photomultiplier detector.

As most biological particles do fluoresce, fluorescence-based detectors are not able to differentiate between pathogenic microorganisms and normally occurring environmental variants. However, an algorithm that detects sudden changes in the biological composition of the environment being monitored can be useful in providing an early warning. The systems on the market can trigger an alarm within 15 to 30 seconds after exposure.

[3] http://www.biral.com/

3.1 Advantages and strengths of commercially available biodetectors

The main purpose of fluorescence-based detectors is to provide a “detect-to-warn” function or to trigger subsequent sampling and analysis using higher resolution methods. In general, fluorescence-based detectors can have good sensitivity, and they are generic in their selectivity so that they are fairly reliable in terms of false negative alarms. The main advantage with this type of biodetector is the extremely fast operational time, often within seconds, allowing real-time surveillance. Some equipment can run continuously since no reagents are consumed and no sample pre-concentration is required.

3.2 Limitations with commercially available biodetectors

The main limitation with existing systems is their lack of adequate resolution. The real-time systems available at present are not specific and they often generate false positive alarms, as there are lots of other particles in the atmosphere that also fluoresce. They cannot distinguish disease-causing microorganisms from naturally occurring organisms. An alarm must therefore be confirmed using other methods or techniques that can carry out specific identification of harmful organisms.

3.3 Products

Fluorescence Aerodynamic Particle Sizer (FLAPS-system), TSI/DRDC/Dycor. Biological Agent Warning Sensor (BAWS) I, General Dynamics. 4WARN system, General Dynamics. VeroTect, Biral. Bio200 BAS, Environics. Instantaneous Microbial Detection (IMD), BioVigilant. Instantaneous Biological Analyzer and Collector (IBAC), ICx. Environics ENVI BioScout.

4 Specific techniques

4.1 Introduction

Targeted approaches are those that answer predefined questions such as is organism X present in the sample? From this it follows that if a negative answer is obtained, the analysis of the sample needs to be repeated to answer the next question, being is organism Y present in the sample? Traditional methods, such as the gold standard method for microbiology i.e. culturing methods, where microorganism concentrations are amplified by growth on suitable substrates or liquid media, are not appropriate in the scope of this report, since culturing is a slow method that takes many hours of incubation before growth can be recorded; not all microorganisms from the environment can be cultivated. Instead, the methods predominantly used at present for rapid (within 60 min) targeted identification rely on major technological and scientific breakthroughs made in the 1970’s and 1980’s, namely antibodies and polymerase chain reaction (PCR).

As already mentioned, though many of the targeted approaches are very powerful in terms of speed, specificity and sensitivity, multiple assays are needed, which adds extra costs and lowers performance in terms of sensitivity and validity of the assays. Most techniques in clinical microbiology traditionally follow a one-target hierarchical approach, with such techniques being categorised in many different ways, including biochemical, immunological, morphological, genetic etc. However, here we have chosen to divide these widely used techniques into immuno-based techniques, DNA amplification techniques and DNA hybridisation techniques.

4.2 Immuno-based techniques

4.2.1 Introduction

Immunoassays may use either polyclonal or monoclonal antibodies, which have a high specificity to an antigen of a given bioagent. Generally, it is the case that polyclonal antibodies have higher sensitivity but lower specificity whereas monoclonal antibodies have higher specificity and somewhat lower sensitivity. The best known and still widely-used standard methods in clinical microbiological laboratories are the different variants of ELISA (enzyme-linked immunosorbent assay). Subsequently, antibodies have been incorporated in miniaturised devices (immunodetectors) that can be used in the field. However, these assays do not provide definitive answers, due to moderate sensitivity, and cross-reactions with closely related microorganisms in the environment, giving rise to false positive reactions. For biodefence detection, for example to detect if a suspicious powder contains a potential biothreat agent, most immunoassays are of the lateral flow assay format similar to a home pregnancy test. Lateral flow assays are usually manufactured in the form of a test strip containing all the assay components encased in a plastic cartridge. The cartridge has a sample window where the sample is applied to the assay strip and a results window where the results are read manually (by eye) or using an electronic optical reader. Depending on the specificity of the immunoassay, false positives can occur if closely related agents are present, such as non-pathogenic relatives of *Bacillus anthracis*. Unlike nucleic acid-based detection devices, the immunoassays have the ability to directly detect toxins, i.e. the actual toxin molecule or protein. A disadvantage is that the devices typically have inadequate sensitivity for bacteria, and they only provide qualitative, not quantitative data.

In a recent evaluation of 12 immunoassays for field screening of *B. anthracis* and ricin, numerous false positives and false negatives were found when testing with powder samples; approximately half of the *B. anthracis* immunoassays evaluated could not detect a concentration of 10^7 spores/mL. The assays performed slightly better for ricin where nine out of 12 detected 0.1 mg/mL of ricin (Bartholomew et al. 2017).

In summary, the sensitivity of immunoassays using the lateral flow assay format is not adequate for field screening of bacteria, considering that, as the infectious doses of most bacteria are in the range 10^2 – 10^5 or lower, the risk of false negatives would be very high. However, for many toxins, including ricin, field screening using immunoassays could be a viable alternative.

4.2.2 Advantages and strengths of the technique

Immunoassays require minimal sample preparation, allow for rapid presumptive identification of both biological organisms and toxins in 15 minutes or less with minimal training and require no sample preparation. The devices are disposable, contain no moving parts, have a long shelf life, and operate over a wide range of temperatures.

4.2.3 Limitations of the technique

Generally, immunoassays have inadequate sensitivity for bacterial and viral pathogens. Cross-reactivity with close neighbours of pathogens in environment samples may also be a limitation (depending on the formulation and batch of the antibody). As with all other phenotypic assays intended for environmental detection of viable microorganisms, the protein target may only be expressed under certain conditions. For example, the FI antigen for *Yersinia pestis* is only expressed at 37°C, which decrease the sensitivity even further in an environmental sample.

4.2.4 Product examples

Examples of commercial products are Tetracor and IMASS (for more examples see[4]).

4.3 DNA amplification techniques

All microorganisms contain genetic material with the total genetic content in a micro-organism being called a genome. Genetic material consists of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). DNA amplification techniques rely on the identification of unique sequences in the genome of the microorganism of interest. By introducing enzymes, singular nucleotides and small tags called primers, which are complementary to the end of the sequence of interest, to the sample, replication of the entire unique sequence will start if it is present.

It is a fairly simple technique to use and understand. The results are produced quickly and it is a highly sensitive and specific technique, although it can only identify the presence or absence of a known microorganism. As it has high sensitivity, any form of contamination, even just a trace amount, can produce a false result. To design primers for a specific region, prior sequence data are required. Primers can anneal non-specifically to a sequence similar to the target DNA, producing a false result.

Quantitative PCR, qPCR, also known as real-time PCR, is a fluorescence-based technique that detects target products using either non-specific fluorescent dye that intercalates into double-stranded DNA, or sequence-specific DNA probes labelled with fluorescent markers. During thermal cycling, the sample is illuminated with light and the fluorescence detected.

A new alternative approach is the development of the LAMP (Loop mediated isothermal amplification) assay that is, unlike PCR, carried out at a constant temperature, ~65°C, and can be easily run without any expensive equipment (Nagamine, Hase and Notomi 2002; Stratakos et al. 2016; Notomi et al. 2015). The assay produces results within one hour and is highly specific, capable of detecting low numbers of target DNA (<10 copies). The isothermal capacity comes from the use of a different polymerase than used for traditional PCR. This enzyme is also less sensitive to inhibiting substances, which lowers the requirements for the sample preparation. The high specificity comes from the fact that this method uses four to six different primers to produce a looped product. One of the drawbacks is the complicated design of the primers. Several LAMP assays have been established and used for detecting different infectious diseases such as malaria, salmonellosis and tuberculosis (Domesle et al. 2018; Kumar et al. 2014; Yamamura, Makimura and Ota 2009).

Another development in the amplification field is the strand invasion-based amplification (SIBA) assay from Orion (Hoser et al. 2014). SIBA is another isothermal assay, carried out at 30°C, with no need for expensive complex equipment. The machine is self-contained and can be run off batteries. This method uses an invasive strand, complementary with the target, which opens up the DNA and gives the primers access to the DNA to start amplification. If the invasive strand fails to bind, no amplification occurs. This extra step makes this method very specific and even capable of distinguishing between closely-related microorganisms. Some assays has been established for this method, targeting e.g. Influenza, *Neisseria gonorrhoeae*, and *Salmonella* spp. (Hoser et al. 2014; K. Eboigbodin et al. 2016; K. E. Eboigbodin and Hoser 2016).

Digital polymerase chain reaction (dPCR) is a recent development in the amplification field. The key difference between dPCR and traditional PCR lies in the method of measuring amplified nucleic acid amounts (Vogelstein and Kinzler 1999). dPCR uses the same primers and probes as real-time PCR but the sample is diluted and partitioned into many

[4] www.cbrnetechindex.com/Biological-Detection/Technology-BD/Immunological-BD-T/Lateral-Flow-Hand-Held-Immunoassay-BD-I

small containers, droplets or chambers. Each partition contains either a discrete number of a biological particle or no biological particles. By counting the number of 'positive' partitions (in which the DNA target motif is detected) versus 'negative' partitions (in which it is not), a very exact quantitative measure of the number of target DNA copies that were present in the original sample can be made (Pinheiro et al. 2012). This makes this an absolute quantitative method and increases its sensitivity.

4.3.1 Advantages and strengths of the technique

The main advantages of the DNA amplification method is its high sensitivity and specificity. The method is robust and thus reduces the requirements for sample preparation. Reagents that can withstand freeze-drying, allowing ready-to-use field applications and assays targeting specific agents, are commercially available. Results can be obtained within 30 minutes and are easy to interpret. Different amplification techniques have different advantages. qPCR allows real-time monitoring of the amplification while LAMP works at a constant temperature of 65°C. SIBA also works at a constant temperature and is highly specific allowing the distinguishing of closely related species. dPCR can carry out absolute quantification (as needed in qPCR) and detect tiny amounts of target in a large amount of background (for example, a rare mutation in a wild-type strain).

4.3.2 Limitations of the technique

In spite of their many advantages, PCR approaches suffer from a number of weaknesses. Their main limitation results from the fact that such an approach is targeted. Only what is precisely being looked for may be identified. Since the bioterrorist threat is associated with a potentially very broad use of pathogens, multiple PCR assays (in the order of tens to hundreds of assays) would need to be developed. Costs are additive, and the assays need to be highly multiplexed due to the usually limited amount of sample, and requirements in terms of sensitivity. In the absence of multiplexing, the sample to be analysed would have to be split into as many aliquots as agents being searched for. This would decrease the global sensitivity of the investigation. Multiplexing produces additional technical challenges, mostly in terms of quality control validation.

4.3.3 Product examples

Examples of commercial products are Orion (based on SIBA)[5], and Loopamp[6].

4.4 DNA hybridisation techniques

4.4.1 Introduction

DNA hybridisation techniques are based on how complimentary strands of DNA/RNA can hybridise with very high specificity. Improvements in computer technology and production techniques have made it possible to analyse 10 – 100 thousand interesting features at the same time (**Figure 3.** Affymetrix GeneChip and Illumina BeadChip designs. (Illustration by Philippe Hupé, distributed under a CC-BY-SA-3.0 license).**Figure 3**). The increase of available sequence data has made it possible to design more sophisticated microarrays by synthesising oligos instead of collecting them. Manufacturing of the first generation of oligo microarrays by Affymetrix was quite complicated, as it required a unique setup. This was later circumvented by a modified technology introduced by Nimblegen which allowed for custom microarrays to be produced in small volumes at much lower costs. Other production techniques, such as inkjet printing by Agilent, have

[5] http://www.oriondiagnostica.com/products/siba/
[6] http://www.eiken.co.jp/en/product/lamp/index.html

been quite successful. They all use tabletop scanners with lasers to analyse emissions to generate results. Currently, different types of oligo microarrays still sell well even though they have been superseded by the introduction of new sequencing technologies.

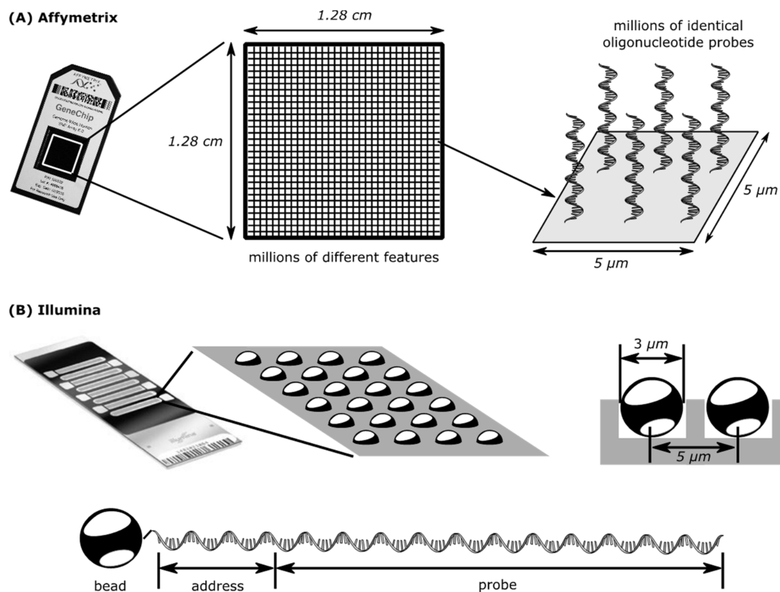

Figure 3. Affymetrix GeneChip and Illumina BeadChip designs. (Illustration by Philippe Hupé, distributed under a CC-BY-SA-3.0 license).

Microarrays are mainly used for clinical samples where the background is known and not complex. For environmental samples, the microarrays can be divided into three major types based on the probes that are used. First, phylogenetic oligonucleotide arrays use rRNA genes for phylogenetic profiling of samples. Those genes are highly conserved and are present in all microorganisms but this also introduces some unique technical challenges. Second, functional gene arrays are useful for monitoring the physiological status and functional activity of potential pathogens. Third, community genome arrays are constructed from known whole genome sequences and can be used to detect the presence of known pathogens.

4.4.2 Advantages

The main advantage of microarrays is that they can be made very specific. The level of specificity can be set to a preferred level for known pathogens. It is also a rather cheap technology, given the data it produces.

4.4.3 Limitations

The major weakness that this technology shares with many others is that it produces an indirect measure of relative concentrations. Signal level is not linearly proportional to the concentration, which can lead to biased interpretations. Another drawback is the limited range of detection where the signal for highly abundant pathogens might be saturated while those with low abundance might go undetected. Highly similar pathogens and unknown close genetic relatives make it difficult to design arrays in which multiple related sequences will not bind to the same probe on the array.

DNA microarrays are a closed technology and can only detect sequences that the arrays were designed to detect. The complication is that microarrays are not sensitive enough for some environmental studies where unknown genetic relatives to known pathogens might give false positives. All this combined makes data mining of microarray results challenging.

The number of genes needed for monitoring on a whole-community scale would extend far beyond the current capacity of microarrays. Finally, microarray equipment is not portable and DNA hybridisation requires at least two hours. The conclusion is that it will not fulfil the set requirements in this report.

4.4.4 Product examples

Examples of commercially available microarrays, focusing on environmental samples, are GeoChip (He et al. 2007) and PhyloChip (Schatz et al. 2010).

5 Broad techniques

5.1 Introduction

Generic or non-targeted analysis aims to detect any microorganism present in the sample (it is not possible to detect everything in practice). A non-targeted method theoretically would allow detection of both known and unknown microorganisms. However, as these methods are normally knowledge-dependent, the quality of compatible reference databases becomes crucial for what can be detected. Here we have included spectral techniques and DNA sequencing as representative examples of the generic approaches that can be used under field conditions.

5.2 Spectral techniques

5.2.1 Near-infrared (NIR), mid-infrared (MIR) and Raman spectroscopy

Thanks to the general technological development in light sources, optical components, electronics, detectors and computer capacity over the last decade, new possibilities for rapid detection have emerged in the field of optical spectroscopy. A new generation of fieldable handheld instruments have been commercially produced that provide rapid, non-destructive, on-site optical detection of bulk chemicals, either as liquids or powders. In principle, a similar methodology can be applied to microorganism detection but, due to a higher degree of sample complexity, the output signals are more complicated to analyse. Most of these compact instruments make use of Near-infrared (NIR), mid-infrared (MIR) and Raman spectroscopy to reveal chemical identity from the molecular vibrational spectrum. At present, both civilian and military sectors routinely use handheld instruments, based on MIR, for attenuated total reflection Fourier transform IR (ATR-FTIR) and Raman spectroscopy. These commercial devices need about 30 s for the measurement and an additional ~30 s to match the acquired spectrum against a reference library containing 10 000 – 100 000 spectra of known chemical compounds. Raman spectroscopy relies on a weak light scattering process and needs no sample preparation at all i.e. in many cases; it could be used with the sample remaining in a container. ATR-FTIR spectroscopy requires the sample to be transferred to a specific ATR crystal. Solid samples have to be pressed onto the ATR crystal to obtain sufficient physical contact for data acquisition. If the sample contains a large amount of water, it can produce very intense peaks for MIR spectroscopy with the risk of overwhelming the spectral signatures from compounds of interest. The reverse is true for Raman spectroscopy i.e. water is more or less invisible in Raman spectra. When it comes to the rapid detection of biological material, this fact is of great relevance since microorganisms naturally have a high water content. NIR spectroscopy and shortwave infrared (SWIR) spectroscopy are less sensitive to water molecules. However, a drawback with NIR and SWIR spectroscopy is that the poor spectral information, in comparison to both Raman and MIR spectroscopy, makes molecular identification difficult. The problem using Raman spectroscopy with biological samples is that they usually contain fluorescent microbial species, often interfering or completely dominating the

recorded spectrum, meaning the more informative Raman scattered photons are dampened and not resolved. This is partly circumvented by the latest handheld Raman instruments utilising longer excitation wavelengths e.g. 1064 nm instead of 785 nm.

5.2.2 Major developmental trends in NIR, MIR and Raman spectroscopy

Examples of real products reaching the market are still very rare; however, the amount of research in this field is intense. NIR, MIR and Raman spectroscopy can be routinely combined with imaging techniques to carry out hyperspectral imaging, meaning information from different parts of the electromagnetic spectrum, including visible light, are combined to reveal the size and shape of an organism simultaneously. This is standard current bench-top technology and incorporates a microscope in the experimental set-up. Studies have been carried out using hyperspectral Raman micro spectroscopy to investigate bacteria and spores non-destructively. Nevertheless, with or without imaging capacity, vibrational spectroscopy has received a good deal of attention over the last decade as development of new rapid analytical tools to analyse microorganisms moves ahead (Dumont et al. 2016).

Most of these studies are related to Raman spectroscopy, especially Surface-Enhanced Raman spectroscopy (SERS) (Figure 4).

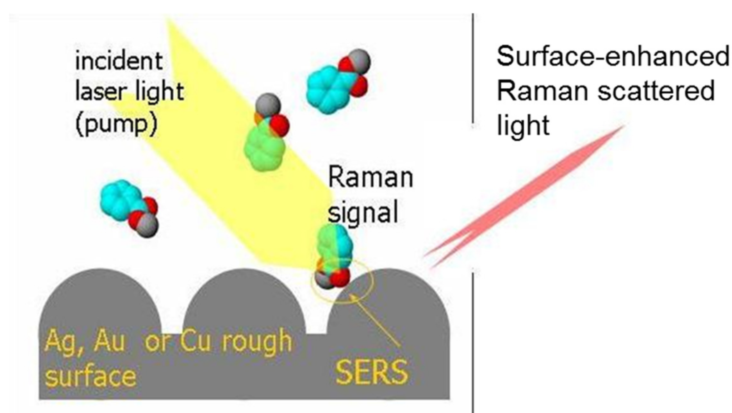

Figure 4. Schematic representation of SERS

In SERS, the analytes must be in close proximity to a nanostructured metal surface (usually Au or Ag) that also quenches eventual competing fluorescence (generated from the background of the complex sample matrices). The compact handheld format, high sensitivity, speed of analysis and multiplexing capability to detect multiple microorganisms simultaneously are all of particular interest (Cowcher, Xu and Goodacre 2013; Jamieson et al. 2017; Farquharson et al. 2014; Liu et al. 2017).

SERS was invented in the middle of the 1970's and was predicted as having a bright future when combined with miniaturised equipment with high sensitivity and specificity for the rapid detection and identification of microorganisms. Such predictions have proven to be overambitious and the market is still waiting for robust cost-effective handheld SERS machines. Over the years, commercial SERS substrates have been expensive and not reusable, but in recent years new alternatives have emerged that reduce the costs without compromising sensitivity and that are suitable for biological compounds such as DNA, proteins and bacteria. Examples are P-SERS™ (Diagnostic anSERS, Inc, USA) and RAM-SERS substrates (Ocean optics, USA); in addition, both companies also manufacture handheld Raman analysers (slightly larger than a cell phone). P-SERS™ is innovatively produced through an ink-jet printing process that reduces the cost considerably. SERS measurements are, in general, rapid (usually 1 – 10 s), with additional time needed for matching accumulated data against a library. The principal of this is exactly the same as that for ATR-FTIR and Raman analyses of bulk chemicals, as mentioned above. One

should remember here that the SERS spectrum does not necessarily overlap with the normal Raman spectrum meaning that a new data library probably has to be created. Another issue to consider is the fact that Raman and SERS spectra of bacteria and spores vary due to growth states and ambient parameters (such as temperature). Therefore, as an alternative approach, specific biomarkers that are linked to certain microorganism and that are abundant and stable have been investigated. A SERS biomarker for *B. anthracis* spores is dipicolinic acid (Cowcher, Xu and Goodacre 2013; Farquharson et al. 2014**)**. Another strategy commonly used is to functionalise the SERS active surfaces for selective binding (Liu et al. 2017).

In summary, many parameters contribute to high spectral variability when analysing microorganisms using SERS (type of substrate, wavelength, condition of growth culture etc.) which limit the inter-laboratory reproducibility as revealed by studies of model bacteria (Dina et al. 2017). To minimise the variability, robust protocols along with compatible and solid reference databases need to be developed before the full potential of the SERS technology can be exploited. However, SERS remains an attractive, highly sensitive method that may lead to rapid identification at the single-cell level in the future.

5.2.3 Portable mass spectroscopy

Mass spectrometry (MS) is routinely used in clinical settings to identify pathogens. For this purpose, a technique called MALDI-TOF MS is used. Whole cells or crude bacterial extracts can be used, which means that a prerequisite is that the microorganisms need to be cultured prior to analysis. The sample is irradiated with a brief laser pulse, the ionised molecules accelerated in an electric field and separated based on molecular weight. The time-of-flight (TOF) of each ion is measured when they reach the detector. The detected signal is then converted to a mass spectrum that can be compared to a database of spectra of known pathogens. In clinical settings, the technique is widespread and well-established in routine diagnostics. The MS analyses are rapid (minutes), but the technique does not work on complex environmental samples and the instruments are not portable (although they could in principle be used in a mobile lab).

There are many other mass spectrometry-based techniques that are already used for identification of the contents of unknown environmental samples. They are mostly used for detecting various chemicals and toxins, but work is ongoing to adapt these techniques for also identifying microorganisms e.g. by identifying marker proteins that differ between closely-related pathogens (Leung et al. 2017; Zhou et al. 2014). There is extensive ongoing work to miniaturise the systems for field use using miniature mass spectrometers and ambient ionisation sources (Chen et al. 2015; Takyi-Williams, Liu and Tang 2015; Ellis et al. 2015). As these are not yet used for identifying organisms in environmental samples, we will not explore MS-based techniques further in this report.

5.2.4 Advantages

The instruments are fast, very easy to use and can be operated with non-professional personnel. Raman-based methods also preserve the sample and, in combination with microscopy-based techniques, can reveal the physical structure of the sample.

5.2.5 Limitations

Reliable reference data have to be accumulated in advance and be incorporated in the compact format, and matching data algorithms have to work correctly. Inter-laboratory reproducibility needs to be improved before SERS technology can reach its full potential. No quantification of the amount of detected target is possible. Only mixtures containing two or three components can currently be resolved with MS techniques, making them unsuitable for environmental applications

5.2.6 Product examples

A portable (19 kg) instrument is available, the Resource Effective Bioidentification System (REBS). Instrument specifications claim that it can detect and identify hundreds of biological agents and pathogens (Battelle, USA) by combining advanced aerosol collection technology with optical spectroscopy (Raman spectroscopy). The instrument is easy to handle and it can be run continuously in automated mode without the need for laboratory support. REBS identifies potential biological threats using spectral matching to predefined reference databases (bacteria, spores, viruses, toxins) within 30 min. The collected samples are examined optically in a non-destructive manner making further analysis to support attribution and medical diagnoses possible.

Examples of miniaturised MS instruments include the Mini10 (Gao et al. 2006), Mini 11 (Gao et al. 2008), and Mini12 (Li et al. 2014) from Purdue University, Tridion-9 GC-MS (Contreras et al. 2008) from Torion Inc. (American Fork, UT), GC/QIT (Shortt et al. 2005) from the Jet Propulsion Laboratory, Chemsense 600 (J. N. Smith, Noll, and Cooks 2011) from Griffin Analytical Technology LLC. (West Lafayette, IN), and the MMS-1000 from 1st Detect Inc. (Austin, TX).

5.3 Sequencing

The DNA-based techniques described above, such as PCR or microarrays, focus on detecting specific DNA sequences (DNA markers) that are unique for a specific pathogen where only organisms with identified specific markers can be detected. However, if the exact DNA sequence of the organisms in a sample can be determined, these sequences can then be matched to known sequences in a reference database. This enables the ability to address questions such as which organisms are present in the sample (and not just whether specific organisms X and Y are present in the sample).

The extent of the reference sequence database limits which organisms can be detected. However, due to the rapid development of DNA sequencing techniques, making sequencing cheaper, faster and easier (see Figure 5 and FOI-R—4217--SE), these databases have rapidly grown in size over the last ten years. Currently, about 5000 eukaryotic (animal, plant and fungal) genomes and more than 12000 prokaryotic (bacterial) genomes are publically available in the NCBI database (ncbi.nlm.nih.gov). Previously, the downstream analysis of the massive amount of data produced by current sequencing technologies was a barrier for many laboratories. Over recent years, the analysis pipelines for many of the standard sequencing applications have matured so that e.g. resequencing of a known genome in order to carry out pathogen identification or identification of sequence variants can, to some extent, be carried out by non-experts. It should be noted that, apart from identification of pathogens, sequencing can also be used for many other applications in the field such as forensic investigations, identification of human remains (e.g. at serious accidents), identification of genetic properties (e.g. virulence factors and antibiotic resistance genes), and identification of artificially modified organisms.

When sequencing an environmental or clinical sample in order to identify organisms, two different approaches can be used. Either all DNA in the sample is sequenced (shotgun sequencing) or a region of the genome that is present in most organisms can be amplified using PCR and then sequenced (amplicon sequencing). Amplicon sequencing requires much less starting material (picogram of DNA) than shotgun sequencing, but takes longer (an extra PCR has to be carried out), and gives lower resolution. Since only a small part of the genome is sequenced, the organisms can usually only be identified down to the genus level. Shotgun sequencing can be carried out more quickly and can identify organisms down to species level, or even isolate, when the database and the accuracy of the reads are of adequate quality. However, shotgun sequencing has higher computational demands and requires more powerful hardware.

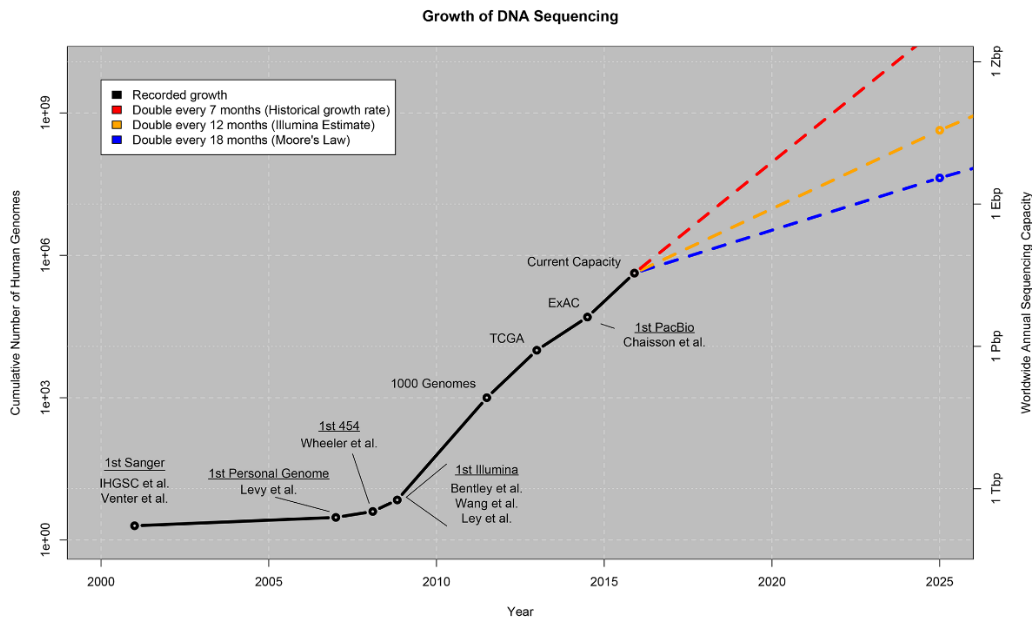

Figure 5. DNA sequencing worldwide. The graph shows the increase in sequencing output over the past 15 years along with three different forecasts for the future. Over the last decade, the development of sequencing technologies has outpaced the computer industry (the so-called “Moore’s Law”) and will soon produce more data than e.g. YouTube and Twitter. Figure and forecast from Zulkifli, Rahim and Lau (2017).

The sensitivity and specificity of sequenced-based techniques are limited by four factors:

(1) whether the genome of the pathogen is present in the database,

(2) how genetically distinct the pathogen is (how much its DNA differs from other pathogens) compared to closely-related non-pathogenic organisms,

(3) how many closely-related organisms are present in the database and,

(4) how accurately the DNA code can be determined by the sequencing technique.

The genomes of most known pathogens are available in the database, but the number of sequenced close relatives varies extensively [Sjödin *et al.* 2013]. In order to improve specificity, more sequences of closely related organisms to the relevant pathogens are, in many cases, needed. The techniques that are used to “read” the DNA sequences in a sample can roughly be divided into two categories: (1) techniques that produce short (50 – 600 nucleotides), highly accurate (<1% error rate) DNA sequences or reads and (2) techniques that produce long (>10 000 nucleotides) but less accurate (5 – 10% error rate) reads. Longer reads cover a larger part of the organism’s genome and can therefore more accurately be assigned to the correct reference genome (the shorter the DNA sequence, the more likely that a particular sequence is shared between several organisms). High accuracy is, of course, better since fewer false matches to the wrong reference genome are made because of errors in the sequencing.

The sequencing market is currently dominated by the company Illumina (Illumina Inc.), whose technology can produce billions of highly accurate (99.9%) reads of 50 – 300 nucleotides. One reason for the popularity of this technology is that it is currently the cheapest per base pair and has the highest output per machine run. For the purposes of this report, the clear drawbacks are that the machines are large and require up to several days to run. IonTorrent (Thermo Fisher Scientific) is another technology that produces high numbers (up to 100 million) of short (up to 400 nucleotides) reads, but the accuracy is much lower than that of the Illumina technology (98%). In contrast to Illumina, the IonTorrent machines are much smaller and faster. A sequencing run can be completed in about two hours. Although these machines would be very hard to run under field conditions (and therefore will not be considered in detail in this report), they would be suitable for well-equipped mobile laboratories. Another advantage is that the machine can be

scaled to produce different amounts of data, greatly reducing the cost and time when the maximum amount of data that could be produced is not needed.

For sequencing technologies that produce longer sequence reads, there are currently two competing technologies on the market. Pacific Biosciences released their SMRT platform in 2013. The machines are very large, expensive and cannot be used in the field, even in a mobile lab. The technology can produce reads (~90% accuracy) of up to 40 000 nucleotides in around half an hour to four hours. In 2016, Oxford Nanopore Technologies released their MinION system, which has a revolutionary design. The accuracy of the sequencing is similar to SMRT sequencing and the read length can be longer (up to several hundred thousand nucleotides). However, the most striking differences are that the machine is not much larger than a normal USB memory stick, extremely portable, cheap (when compared to other sequencing machines), powered by a computer USB port and delivers results in real-time as the sequencing proceeds. The portability and extremely rapid delivery of sequencing results makes it potentially very suitable for rapid identification of pathogens in the field. Since the MinION system is really the only fieldable sequencing platform, we will now focus on this technique.

5.3.1 MinION

Most sequencing technologies use the enzyme that normally copies DNA in living cells and then reads the sequence of nucleotides as they are added by the enzyme. This limits the theoretical upper limit for read lengths as the enzyme gradually degrades. The MinION system uses an entirely different process to read the nucleotides in a DNA molecule (Figure 6). Here, DNA is forced through a nanopore in a membrane using an electric current. When different nucleotides pass through the pore, they disrupt the ion flow through it. The disruptions in the ion flow are detected using sensitive sensors and the information is passed in real-time to the computer that the MinION is connected to. The computer can then translate the ion flow data back into a DNA sequence. The machine has 512 pores that can simultaneously read one DNA strand each at a rate of several hundred nucleotides a second. A powerful laptop can both control the MinION device and translate the ion flow signal into a sequence. However, the translation into a sequence is computationally demanding and for this translation to keep up with the speed of a MinION machine running at full capacity, more powerful computational servers are currently needed. The algorithms are constantly improving and tests to run the computations on a graphics card (that can handle more parallel computations) instead of using the standard processor are underway. Once the DNA sequence has been obtained, each sequence needs to be compared to a reference database containing the genome sequences of relevant organisms. This step can easily be carried out on a standard laptop.

Figure 6. The MinION device (Oxford Nanopore Technologies).

5.3.2 Advantages and strengths of the technique

The main advantage of sequencing over most other technologies is that any organism or mix of organisms can be identified in a sample without prior knowledge of the sample. Instead of asking if organisms X and Y are present, one can just ask what organisms are present. When compared to other sequencing technologies, the MinION system is small, portable, robust, extremely fast and produces the longest reads (up to almost a million nucleotides). This makes it ideal for the purpose covered in this report. It is also similar to the IonTorrent technique, scalable for different purposes that require different amounts of

data. The fact that the machine is much cheaper (~100 to 1000 times) than other competing machines is also an advantage.

The MinION system has already been used in the field in places such as Antarctica (Johnson et al. 2017), the Tanzanian rainforest (Menegon et al. 2017) and in microgravity (McIntyre et al. 2016), clearly showing the robustness of the technique. It should be noted that these studies did not focus on speed and most of the data analysis was not carried out on-site. FOI has worked with early versions of the MinION system in collaboration with Oxford Nanopore Technologies since 2014 and has evaluated its capabilities for rapid field identification. In 2016, FOI tested a workflow developed in-house that only used very simple equipment (requiring no mains power) and that could easily be brought into the field. The equipment consisted of a MinION device and a standard laptop. Using this set-up, *Bacillus atrophaeus* (historically known as *Bacillus globii)* spores could be correctly identified within 12 minutes of opening the sample container.

5.3.3 Limitations of the technique

Currently, the main limitation of this technique is the relatively high error rate (~5 – 10% error rate) and the large amounts of pure DNA required (a few hundred ng). Both the error rate and the amount of input material required reduce with new updates of the technique, but both need to be lowered further for the technology to reach its full potential. At present, there are kits that in ~30 min can amplify DNA so that the required sample volumes can be reduced at least 10-fold. However, this will add valuable time to the analysis and requires additional PCR equipment.

The error rate is somewhat compensated by the length of the reads, but false positive signals from closely-related organisms can still occur when sequencing complex samples containing many organisms.

6 What techniques will be available in five to ten years?

In this section**,** we speculate on what will be available as fieldable products in the near (within five years) and more distant (up to ten years) future. Most of the techniques described have already been shown to work under ideal (laboratory) conditions, but much work remains to make them robust and reliable enough to withstand field conditions.

6.1 Five-year horizon

Several new molecular techniques for detecting specific DNA sequences are on the horizon. SHERLOCK is a new method but not yet a commercial product. It uses CRISPR (highly specific guide RNAs) to direct Cas13a to specific DNA targets. Cas13a exhibits promiscuous ribonuclease activity on target recognition. Once targeting is achieved, Cas13a cleaves a supplied reporter that releases a dye that can be detected. This method can detect the target DNA with high specificity (single-base mismatch specificity) at extremely low concentrations (attomolar) (Gootenberg et al. 2017). Rapid and PCR-free DNA Detection by Nanoaggregation-Enhanced Chemiluminescence is another example which has a limit of detection down to 260 fM (260×10^{-15} M). The detection can be completed within 22 minutes using only a portable luminometer (Singh et al. 2017). Other approaches to rapid detection of pathogenic bacteria may involve the capture of bacterially-released volatile organic compound profiles (Lough et al. 2017; Saviauk et al. 2018).

Sequencing DNA is very effective when the goal is to identify organisms in a sample at high resolution. However, the presence of DNA does not reveal whether the identified organisms are biologically active or not. In other words, the organism can be dead even if its DNA is present. Instead of sequencing DNA, it is possible to sequence RNA, which

rapidly degrades when the organism dies. The RNA can also tell what genes are active and therefore which state (actively replicating, toxin producing, dormant etc.) the organism is in. Sequencing RNA usually requires the conversion of RNA to cDNA before sequencing. This is too difficult and time-consuming to carry out in the field. However, there are protocols for direct RNA sequencing using the MinION platform (A. M. Smith 2017). These protocols are still in development and are not yet robust enough to be considered for identification purposes and field use. We estimate that this is likely to change within five years.

Detecting a low abundance of organisms in samples with a high background of other organisms is challenging for current sequencing techniques. Large amounts of sequencing data need to be produced for low abundant DNA to be picked up by a sequence-based analysis. There are methods to enrich the DNA from specific organisms in a sample or deplete DNA from others, but these techniques usually require a laboratory environment and take several hours to complete. Using nanopore sequencing, the sequence can be read in real-time so that a partial sequence can be analysed before the entire DNA molecule has passed through the nanopore. The polarity of individual pores can be controlled by the MinION device, meaning that if the initial sequence coming through the pore can be analysed rapidly enough, the DNA molecule can be backed out of the pore if it belongs to an unwanted organism. This type of selective sequencing has been shown to work on simple samples when only monitoring a few pores (Loose, Malla and Stout 2016). When the computational methods and hardware improve, it will be possible to use inclusion and exclusion lists of organisms, greatly improving the sensitivity when sequencing complex samples. An example would be when looking for low abundant pathogens in clinical samples where the vast majority of the DNA is of human origin.

In the coming years, there will be a focus on automated sample preparations systems, such as VolTRAX (Oxford Nanopore, https://nanoporetech.com/products/voltrax). Combined with MinION sequencing, it would give an almost fully automated sample sequencing analysis system, running from sampling to reporting.

6.2 Ten-year horizon

Portable Mass Spectrometry-based machines are being developed. MS-based techniques may become highly relevant for continuous monitoring of pathogens in the environment if proteins with spectra that can resolve sub-species differences for relevant pathogens are identified, and high quality reference data can be produced (along with standardised sample preparation). The advantage compared to DNA-based techniques will be, for example, that the activity of organisms and the presence of toxins can be detected.

Over a ten-year horizon, it will likely be possible to sequence single bacterial cells in the field. Methods, such as crosslinking (Marbouty et al. 2017), nano-droplets (Woyke, Doud, and Schulz 2017) and similar already exist, but they are far too complicated to carry out in the field. Such techniques make it possible to link properties (antibiotic resistance, pathogenicity factors, plasmids etc.) to specific organisms so that a complete picture of a pathogen's characteristics can be determined. This is not possible with current techniques.

Nanopore sequencers are considered to be a disruptive sequencing technology but still need sample preparation before sample analysis. In the future, this may change by developing methods, which require no protein components to be involved in the sequencing and bypassing library preparation so that biological samples can be sequenced directly. Together with the introduction of solid-state pores, such a system could be used for continuous high resolution surveillance.

As sequence databases grow, the identification of organisms using sequencing will greatly improve. Once database coverage is extensive enough, we will be able to not only identify organisms at the species level, but also by looking at regional population differences be able accurately to predict the geographical origin of samples. This will greatly enhance forensics investigations. To some extent, this is already possible for a very limited number

of well-studied organisms. By analysing the sequence data, researchers are also improving their prediction of phenotypes (e.g. physical appearance) based on sequence. It is already possible accurately to predict gender, hair colour and approximate ethnicity of people based on their DNA sequence (see Kayser (2015) and references therein). In one recent study, researchers were able quite accurately to reconstruct the faces of people only based on their DNA sequence (Lippert et al. 2017).

A growing concern is the future creation of novel pathogens using synthetic biology. Identifying these will be a challenge. Even more difficult will be predicting what properties and functions these organisms and the proteins they produce might have. In order to handle this future threat, we need better and more extensive reference DNA databases as well as better functional prediction tools. Both these will greatly improve in the next 10 years, but it is hard to predict if this improvement will keep up with our abilities to create novel biological functions and organisms that have never existed in nature before.

7 Concluding remarks

Although this report focuses on the detection of pathogens, there are other areas where the development of fieldable techniques for identification of organisms is taking place. Traditional surveillance of biodiversity and endangered species is now gradually being replaced or complemented by DNA-based analysis of environmental samples (so-called eDNA analysis). Products are being developed that are able to detect organisms rapidly under field conditions. Another field where a good deal of effort and resources are being invested is Point-Of-Care diagnostics. Here the concept is to bring the analysis to the patient instead of sending samples for time-consuming analysis elsewhere, in order to design a correct treatment more rapidly. The focus is not only to identify a pathogen, but also to identify the properties of that pathogen (e.g. antibiotic resistance). Clinical samples are less complex than environmental samples, but have high levels of human-related background. Since the objectives are very similar, it will be important for organisations focused on detection of bioagents to follow the development in these two fields closely.

The ideal bioagent detector would be an instrument that autonomously and continuously monitors the environment, identifying all organisms (bacteria, fungi, viruses etc.) and biological products (e.g. toxins and virulence factors). Ideally, the activity and properties (e.g. antibiotics resistance) of the organisms would also be determined. It would also quickly analyse the results on-site and deliver informative reports along with recommended actions when the presence of any potentially dangerous material was detected. To fulfil all of the above, the DNA, proteins and metabolites of the sample would have to be analysed and it would need to be analysed at the single-cell level. Although this could in principle be done at high cost in dedicated laboratories at present, it is unrealistic to suppose that it will be possible to carry out such analysis using portable field equipment within the next 10 years. The information from analysing proteins and DNA is, in many respects, similar when it comes to identification. Protein analysis has the advantage that it can detect the presence of some toxins and virulence factors, where DNA analysis can only detect the ability to produce such substances. However, DNA has a higher resolution in discrete levels (A, C, T and G) when it comes to separating organisms at the species or sub-species level, something that is sometimes crucial. For this reason, and because several very small and portable devices are coming onto the market, we predict that for the near future, DNA-based detectors will dominate this field.

The analysis of the, sometimes, very complex data that comes out of the machines is becoming easier as software and computers are developing quickly too. However, rapid interpretations in the field will unlikely take advantage of the full dataset that is produced. Deeper analysis of the data by a specialist in many cases can reveal more information about the sample. Optimally, the data should be quickly processed in the field in order to assist rapid decisions regarding, for example, protective measures. At the same time, the full dataset could be wirelessly transferred to experts located elsewhere. More complete

analysis could reveal details about e.g. virulence properties of the detected pathogen or likely origin, relevant for forensic investigations. Even if rapid analysis and interpretation can be carried out in the field, a set-up that combines handheld analysis with data transfer and reach-back capabilities will likely be the best approach for the foreseeable future.

Table 1. Fieldable identification techniques.

Technique	Broad	Can be used in	Minimal analysis time	Sensi-tivity	Handles complex samples	Can detect toxins
Immuno-based	No	Field	15 min	Low	Well	Yes
DNA amplifica-tion	No	Field	20 min	High	Well	No
Spectral	Yes	Field	1 – 30 min	High	No	Yes
Sequencing	Yes	Field/mob ile lab	15 min	Low	Medium	No

Even if the technical development is very rapid in the area of pathogen detection, the current techniques presented in this report all have different strengths and weaknesses, see Table 1. Therefore, a combination of techniques will still have to be used for critical operations where continuous sampling needs to be combined with high-resolution identification.

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