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POINT-OF-CARE WHITE PAPER | April 2022

Rapid AST using nanofluidics

How to perform real-time Antimicrobial Susceptibility Testing (AST)

Why should we care about antimicrobial resistance?

For many years, the World Health Organisation (WHO) has issued warnings about the increasing threat posed by antimicrobial resistance (AMR) [1]. The emergence and rapid spread of antimicrobial-resistant bacteria poses a real danger: it can be observed in nearly all countries across the world and affects all kinds of patients. An innate characteristic of bacteria is their ability to adapt to their environment. However, the pace of their adaptation to antibiotics and development of resistances is closely related to the overuse of these drugs. This pace of adaptation has become a matter of concern. Due to the widespread use of broad-spectrum antibiotics, improper use of antibiotics for non-bacterial infections, insufficient diagnostics and a lack of stewardship programs, AMR is now an ever-increasing phenomenon [2, 3].

Voices sounding the alarm have become louder in recent years – and with good reason. If the antibiotics currently available to us lose their efficacy in treating bacterial infections, simple medical procedures could become life-threatening. In other words, infections that are currently treatable and considered manageable could run out of control, weakening the power of available medicines. It is our duty to rationalise the use of antibiotics and use tools to avoid their misuse before their efficacy declines and bacteria win the battle [4]. The rationalisation of the use of antibiotics is also relevant in terms of their side effects: antibiotics also attack beneficial bacterial flora in our bodies, which might increase the patient's vulnerability to new infections.

Last but not least, the pharmaceutical industry and biotechnology companies are investing less heavily in the research and development of new antibiotics [5].

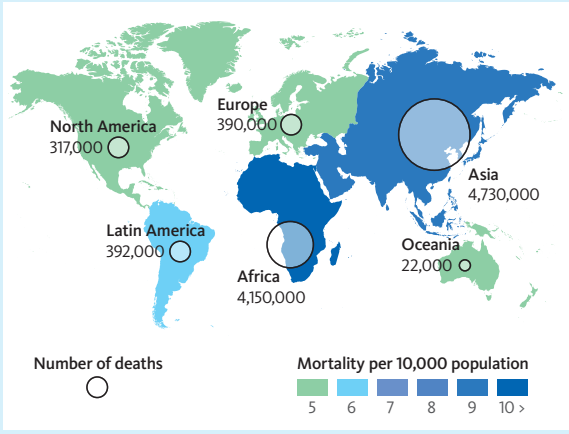

Fig. 1 *AMR knows no borders. If globally effective measures are not put in place soon, AMR could be the cause for 10 million deaths worldwide by 2050 [2].*

The role of diagnostics in fighting AMR

Diagnostics play a major role in the rational prescription of antibiotics. Well-known antimicrobial susceptibility tests (AST) are reliable tools for the phenotypic determination of resistance to antibiotics [6]. Several solutions to support AST performance are available on the market.

Bacterial cultures are usually performed in specialised microbiology laboratories by trained staff. Most of the methodologies available to us today have been applied for decades. They are based on generating antibiograms by monitoring the capacity of bacteria to grow in the environment to which they are exposed (i.e. exposure to antibiotics). One of the critical points in acute situations is the need for an overnight culture prior to the AST itself. This delay in the availability of the AST results translates into late clinical diagnosis, which in turn leads to the recurrent use of empiric therapy to deal with suspected bacterial infections. Another consequence of this diagnostic prolongation is the use of broad-spectrum antibiotics to cover the possibility that multi-drug resistant bacteria is causing the infection. It has been estimated that approximately 30 % of prescriptions at doctor's offices and emergency departments are unnecessary, which could be addressed if faster diagnostic tools were available [7].

The development of novel methods for the performance of AST with focus on reducing the time-to-results is key to advanced diagnostic tools. Without impacting on quality, these solutions would provide the required guidance for the treatment and, eventually, prescription of antibiotics.

Current challenges of AMR and available AST methods

- Ever-increasing resistance rates, causing antibiotics to lose their effect and reducing treatment options.
- Too few new antibiotics are being developed.
- Current AST methods entail lengthy analysis time and require in-depth expertise.
- Interpreting AST results from a microbiology lab requires specific and up-to-date knowledge.
- The lack of diagnostic alternatives makes empiric therapy standard in many settings.
- Empiric therapy relies on prescribing antibiotics that work for the patient based on statistical risks, which should be avoided to fight AMR.

Fig. 2 *Current AST diagnostic methods often involve time-consuming methods that also require technical expertise.*

Genotyping versus phenotyping

Genotypic AST uses methods including sequence-specific PCR amplification, taking advantage of its high sensitivity and specificity to identify resistance genes. However, genotypic testing has some drawbacks. Not only does it require advanced expertise and specialised equipment, its focus on the genetic sequence of bacteria means that it cannot detect new resistance mechanisms. Furthermore, the presence of resistance genes does not always lead to a phenotypic (actual) resistance.

In order to produce results that offer the maximum added value for the attending physician, efforts have shifted towards the development of new phenotypic AST methodologies. The advantage of the phenotypic approach over genotypic analysis is that it does not need to target any characteristic known beforehand (such as a resistance gene), instead relying on the behaviour of the bacterial strain in a real environment. By testing the actual resistance profile of bacteria present in the sample, it is possible to produce actionable results, based on the particular strain causing the infection.

Genotypic vs phenotypic characterisation of bacteria

The genotype is the genetic material of an organism, i.e. the genes that make up its genome. The phenotype refers to the set of observable characteristics of an individual, which depends on the genotype and the environment.

While genotypic analysis methodologies target the identification of genes within the genome of an organism, phenotypic methodologies analyse the characteristics actually expressed by the organism. Nanofluidics-based AST, like culture-based methods, can therefore be classified as phenotypic analysis methods since the result depends on the response of bacteria when exposed to certain antibiotics – their actual resistance profile.

Nanofluidics meets AST

Conventional AST methodologies require colonies of bacteria to perform AST. The result can only be read when a colony of 10^7 cells has formed and, given the natural growth and division rate of bacteria, typically implies overnight cultures. The use of nanofluidics allows the measurement of bacterial growth before a bacterial colony forms, measuring growth as length extension of individual cells instead of the time until they grow and divide to form a visible colony.

This disruptive way of performing bacterial cultures makes it possible to bypass the limitation of bacterial growth rate in conventional cultures. In other words, using nanofluidics enables us to generate results in the real-time scale of bacterial cell growth, not in that of macroscopic bacterial colonies. This is the key to the analysis speed of the system.

This way of measuring bacterial growth has also the advantage of detecting the existence of bacteria in a sample. Monitoring individual cell growth makes it possible to quickly determine bacteriuria in a sample – faster than with conventional cultures. The impact of ruling out negative bacteriuria samples in terms of sparing antibiotic treatments is clear.

The application of nanofluidics in microbiology represents a new paradigm in bacterial culture and opens the door to performing AST on a single cell. By miniaturising the bacterial cultures, we are able to reduce the time until the effects of antibiotics on bacterial cells are observed. This concept has tremendous potential to reduce the time-to-results and brings us closer to the creation of diagnostic tools capable of providing rapid assistance in the diagnosis and treatment of bacterial infections [8].

How does the system work?

The core of the AST analysis system is a nanofluidic chip that contains an array of traps (nanochannels), partly closed at one end and open at the other, where they connect to a central flow channel [9]. The sample flow is pushed through the central channel and the individual traps, which are randomly populated with the bacteria in the sample. Only one bacterium, or a very limited number of bacteria, remain trapped in each nanochannel, as the flow through the nanochannel is partly blocked when it has trapped a cell.

After this loading step, the flow of sample is substituted by an intake of growth medium. Analogous to the flow of sample, the growth medium flows through the central channel and the individual traps, now providing a suitable growth environment for the bacteria. The individual bacterial cells initially trapped in the nanochannels will grow along the channel. Since the width of the nanochannel only allows one bacterial cell to fit in, cell growth causes the formation of a straight line of cells, which eventually fills the totality of the nanochannel. This geometry provides the basis for the measurement of bacterial growth dynamics, which is performed at the single cell level.

In a subsequent step, different conditions are generated in different groups of nanochannels. Each set of nanochannels, consisting of several hundreds of individual traps, can be exposed to different conditions, i.e. different antibiotics and/or concentrations. This can be achieved by means of a complex network of channels, with each set operated independently from the others. In this step, the growth medium flows through reservoirs with different antibiotics dried in at different concentrations, creating differing conditions in each set of nanochannels. If a bacterial strain is

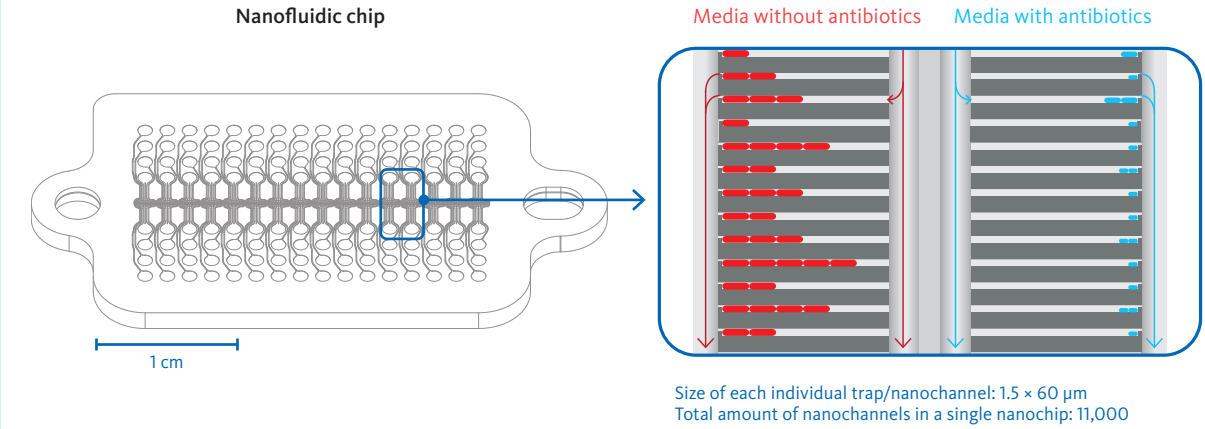

Fig. 3 *Sample flow through the nanochannel system. Different conditions (represented by the different colours) are applied to each subset.*

susceptible to the antibiotic to which it is exposed, the cells will show reduced growth or will even lyse. Resistant bacteria will grow and multiply, thus filling the nanochannel. The extent to which nanochannels are filled at different concentrations is conditioned by the degree of resistance of the bacteria to the antibiotic.

The system facilitates parallel testing of several antibiotics at various concentrations, recording bacterial growth rate in the individual traps and generating multiple individual recordings for a single condition. This data is then processed to generate an average growth impact for many different bacteria growing in the same condition. The impact is compared to that of reference conditions without antibiotics, since different samples will have different native growth rates irrespective of antibiotic treatment. Using data from different antibiotic concentrations then makes it possible to calculate the clinical breakpoint of the bacteria.

Bacterial growth in such nanofluidic channels is monitored by optical detection, using phase contrast microscopy. The nanofluidic chip moves beneath the microscope to generate a set of images covering the thousands of individual traps that constitute the analysis area, a few hundred at a time. The process is repeated every 30 seconds, which allows the creation of a video to monitor individual cell growth.

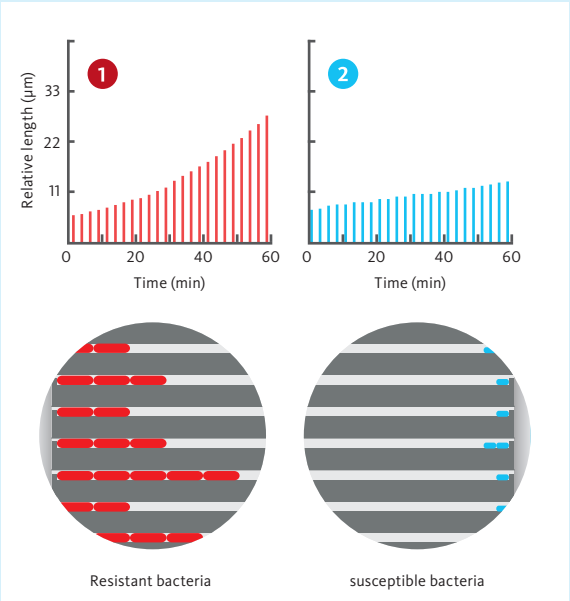

Fig. 4a *Resistant bacteria grow, following an exponential trend when they are resistant to the antibiotic applied (1), while their growth is much reduced, or even stopped, if the strain is susceptible to the antibiotic tested (2).*

Fig. 4b *Bacteria growing in the nanochannels. Resistant bacteria grow along the nanochannel until it's completely filled. Susceptible bacteria lyse or show a very reduce growth rate.*

The data processing algorithm is key for the interpretation of the results. Continuous monitoring of cell growth at single cell level dramatically accelerates this analysis compared to conventional bacterial cultures. Instead of waiting for a bacterial colony to grow, nanofluidics allows a sort of real-time growth monitoring. This unique feature makes it possible to generate clinically relevant diagnostic results within minutes – which other AST methods cannot achieve.

Key advantages of nanofluidics in AST diagnostics

- AST from single cells
- Growth detection in real time
- Fastest possible AST – as fast as the biological response to the antibiotic
- Reduced footprint compared to conventional microbiology
- Fully automatable analysis: no expertise needed for use and independence from operator
- Lab-on-a-chip concept: small size, potential for POCT

Challenges of using nanofluidics

Some attempts to incubate bacteria in microfluidic and nanofluidic set-ups are highlighted scientific literature [10]. This innovative way of performing cell cultures presents some challenges due to the behaviour of fluids in the nanofluidics scale and the analysis of individual cells. One of those limitations is the difficulty of capturing individual cells in the nanofluidic system. Literature also provides some solutions for this, most of which involve pre-analytical steps as pre-concentration of bacterial cells. The second limitation is the irregular concentration of antibiotics during the analysis if a constant supply of fresh medium cannot be maintained. The final key constraint is the difficulty in measuring cellular growth rate using optical detection methods.

The miniaturisation of the system requires a high degree of automation, which presents certain limitations in terms of identifying some bacterial species. While the system can be configured to offer a solid classification of certain bacterial species, it is not easy to generate an environment where all potential pathogens can grow. Several factors, such as cell size or required growth media conditions, still require the analysis of complex samples in a microbiology laboratory. The knowledge and variety of tools available to the microbiologist will still play an important role and nanofluidics will not replace it. Extensive comparison with reference methodologies is required to ensure a safe application in the clinical context.

Due to these limitations, the implementation of a nanofluidics-based system for the performance of AST is far from a trivial task. The system we are presenting has been designed to overcome those challenges and lays the foundations for a diagnostic device using this highly sensitive and fast technology. The advantages of this system lie not only in its ability to capture individual cells, but also in the precise monitoring of the cells' growth rate in terms of cell division and cell growth. This system also avoids the need for sample pre-treatment.

These are fundamental features to bring the technology closer to a medical diagnostic device. This technology provides a key tool to make AST available to locations outside the microbiology lab, which represent a significant increase in the quality of diagnostics for a considerable number of patients.

Looking to the future

How can nanofluidics-based AST be applied to everyday practice? This technology will be the basis for innovative lab-on-a-chip diagnostic devices. When combined with artificial intelligence or knowledge-based software, this will allow the integration of clinical microbiology knowledge to some degree. By developing a fully automated system, it is possible to remove the dependence on the operator, typical of complex analysis steps in conventional AST methods. In essence, this nanofluidics system can, to a certain extent, bring dedicated microbiology lab methods onto a silicon chip.

Nonetheless, one thing should be clear: these technologies are certainly not intended to replace microbiology laboratories. Certain factors limit its potential, including the many complexities in microbiology procedures and biological variations in species. The vast number of potential pathogens, their different behaviours and multiple treatment possibilities narrow the usage to dedicated applications.

Yet, the key advantages outlined above make this technology an outstanding candidate for the creation of a new class of point-of-care testing (POCT) devices. Reduced size, operator independence, ease of use and fast results are classic features of POCT diagnostic instruments. The union of nanotechnology and data analysis makes fast AST available, which in turn offers new opportunities at the point of need.

For everyday infections, the possibility of a near-patient testing device provides a unique tool to select antibiotics tailored to each infectious episode. Making this information available to the treating expert and so close to the patient is key in the fight against AMR. Fast AST results will allow us to use antibiotics with elevated resistance rates in cases where they remain effective, thus prolonging their useful life, as well as conserving reserve ('last-resort') antibiotics and decreasing drug costs.

To understand the potential of nanofluidics in the AST diagnostic landscape, think about some infectious diseases that, for different reasons, attract considerable attention. For instance, consider the critical value of time in the diagnosis of sepsis, where every minute counts. In another context, one could see a considerable impact in public health, if cystitis – the most common urinary tract infection and one of the main diseases leading to antibiotic prescription – were diagnosed using such technology in a near-patient setting.

Sysmex is committed to developing the future of diagnostics and providing healthcare with advanced tools that offer clear added value for the clinician and for the patient. Having nanofluidic instruments in a doctor's practice would appear a game-changing idea. Indeed, it is only by changing the rules of the game that we can take diagnostics to the next level.

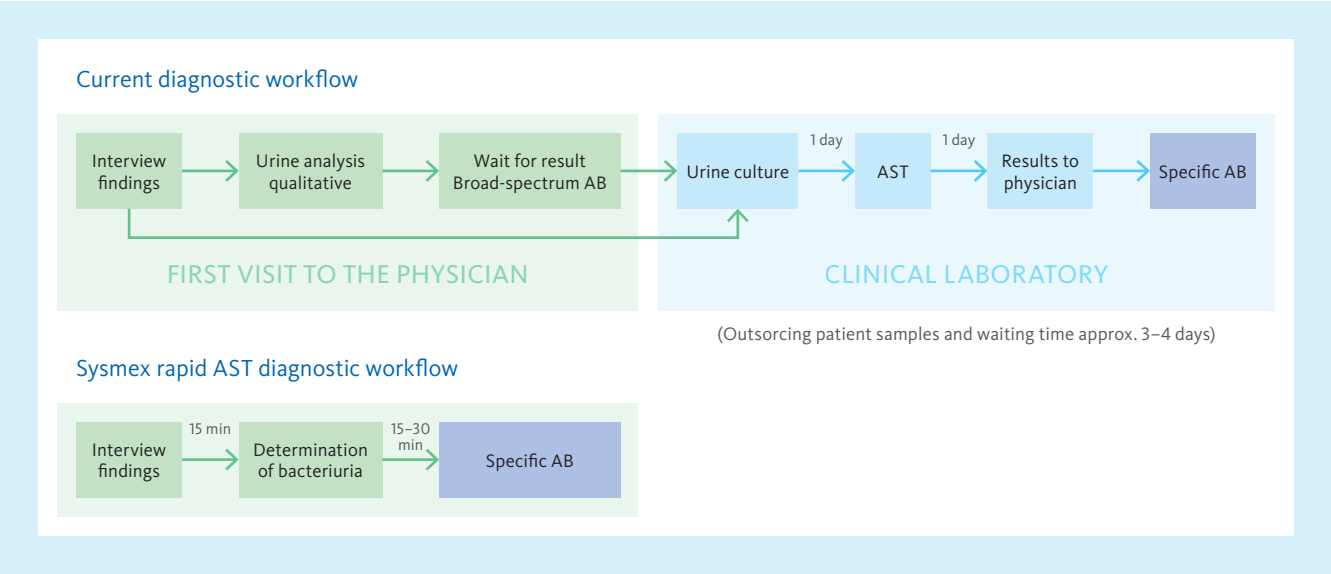

Fig. 5 *One potential application of this technology could be the rapid AST of urine samples. The current UTI diagnostic workflow requires sending the urine culture to an external laboratory. Overnight incubation steps are nowadays mandatory. Taking this innovative technology into a POCT device could allow shortening the time to diagnostics to less than one hour.*

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EN.N.05/22