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Culture-Independent Diagnostics for Health Security

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Introduction

The past decade has shown considerable development in the diagnostic application of non-culture methods, including nucleic acid amplification-based methods and mass spectrometry, for the diagnosis of infectious diseases. The implications of these new culture-independent diagnostic tests (CIDTs) include bypassing the need to culture organisms thus potentially impacting public health surveillance systems, which continue to utilize isolates as the basis of their surveillance programs and to assess phenotypic resistance to antimicrobial agents. CIDTs may also affect the way public health practitioners detect and respond to a bioterrorism event. In response to a request from the Department of Homeland Security, Los Alamos National Laboratory and the Centers for Disease Control and Prevention co-sponsored a workshop to review the impact of CIDTs on the rapid detection and identification of biothreat agents. For this purpose we considered diagnostics in broad sense to include true clinical diagnostics as well as detection assays used in biosurveillance systems. The workshop was held concurrently with the March 8-13, 2015 Gordon Research Conference on Chemical and Biological Terrorism Defense to draw upon subject matter experts attending this meeting. Additional experts from the fields of nucleic acid amplification technologies, mass spectrometry, antibody-based diagnostics and next generation sequencing were also invited. A list of workshop participants is in Appendix 1. To address the impact of CIDTs on the ability to detect and identify the agent of a biological event, four panel discussions were held that covered nucleic acid amplification-based diagnostics, mass spectrometry, antibody-based diagnostics and next generation sequencing. Exploiting the extensive expertise available at this workshop, we identified the key features, benefits and limitations of the various CIDT methods for providing rapid pathogen identification that are

critical to the response and mitigation of a bioterrorism event. After the workshop we conducted a thorough review of the literature, investigating the current state of these four culture independent diagnostic methods. This report combines information from the literature review and the insights obtained at the workshop. It is clear from this review that despite the many recent advances in culture-independent diagnostics that a single technology does not yet exist that can meet the majority needs of both clinical diagnostics and environmental biosurveillance. Many CIDTs are well suited for particular diagnostic or detection questions and this review may help direct the reader to these best use application spaces. Features of an ideal platform for clinical diagnostics and environmental biosurveillance are provided in the summary.

Nucleic Acid Amplification-Based Diagnostics.

The polymerase chain reaction (PCR) and real-time PCR are the most widely used nucleic acid amplification based methods for diagnostics. However, in this session, a broad range of nucleic acid amplification-based technologies were discussed; including self-sustained sequence replication (3SR), nucleic acid sequence based amplification (NASBA), strand displacement amplification (SDA), ligase chain reaction (LCR), transcription-mediated amplification (TMA), rolling circle amplification (RCA), loop mediated isothermal amplification (LAMP), smart amplification (SmartAmp), helicase-dependent amplification (HDA), multiple displacement amplification (MDA), single primer isothermal amplification (SPIA), and recombinase polymerase amplification (RPA). Many of these alternatives to traditional PCR amplify nucleic acid targets under isothermal conditions, eliminating the requirement for thermal cycling and potentially simplifying the incorporation of diagnostics into point of care devices. We refer the reader to several reviews that provide details of these alternative methods (1-6).

Nucleic acid amplification-based diagnostics or Nucleic Acid Amplification Tests (NAATs) are very well suited for environmental surveillance, clinical diagnostics, food and water safety and other situations where DNA or RNA analytes provide suitable targets for amplification and detection. NAATs are highly scalable from single assays to hundreds or even thousands of assays with currently available instrumentation. Analytical sensitivity is excellent, routinely ranging from 10 to 50 genome copies for most nucleic acid amplification based methods, and even to below single copy sensitivity for digital PCR methods employing thousands of replicate reactions in parallel (7).

Clinical sensitivity is different than analytical sensitivity and is dependent on the amount of agent or its nucleic acids in the sample as well as the sample type. Clinical sensitivity for NAATs can be higher than culture-based methods in some matrices, such as the detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in urine (8), and for the detection of respiratory viruses in nasopharyngeal secretions (9, 10). PCR diagnosis of extrapulmonary tuberculosis has been shown to be more sensitive than acid-fast smears examined by fluorescent microscopy (11). However, given NAATs inability to discriminate between live and dead organisms and the variability in persistence of nucleic acids from dead pathogens, clinical interpretation of the state of an infection is not straightforward or even possible based solely on the results of these diagnostics (12-14). The question of pathogen viability also impacts interpretation of NAATs in food, water and environmental samples. Alternative strategies have been developed to provide a molecular assessment of microbial viability (15), but these are more complex and are not currently incorporated in commercially available NAATs for pathogen diagnostics.

Specificity is also a strong point of nucleic acid based diagnostics, which can readily provide species and/or strain level detection or discrimination without detecting near neighbors. However, specificity is entirely dependent on the design of primers and probes, which is in turn dependent on the availability of sufficiently representative genome sequences of the targeted pathogen and its most closely related near neighbors. Confidence in specificity requires experimental demonstration of assay specificity through testing of diverse screening panels to validate performance. PCR performance standards developed to support government programs include the Standard Method Performance Requirements (SMPRs) developed by the Stakeholder Panel on Agent Detection Assays (SPADA) and assay performance standards developed by the Public Health Actionable Assays (PHAA) program and Federal Standards for Assay Performance and Equivalency (FSAPE) (16).

Among nucleic acid amplification based diagnostic methods, real-time PCR (also known as quantitative PCR or qPCR) is the "gold standard" for quantitative analysis, offering quantitative results over a broad dynamic range of up to 8-orders of magnitude. Relative quantitation among samples is straightforward using the $\Delta\Delta C_t$ method (17, 18). Absolute quantitation with real-time PCR requires use of a standard curve. End point amplification methods, including those based on conventional PCR (e.g. Luminex xTAG RVP and Genmark Respiratory Viral Panel assays) or isothermal amplification methods, are not reliably quantitative over the same range and have reduced sensitivity compared to 5' nuclease real-time PCR (19).

The portability of nucleic acid amplification based diagnostics is dependent on both the technology and the platform used. Real-time PCR is performed in clinical or research laboratories using bench top instruments that cost in the vicinity of $50,000 (without automation)

for sample preparation, and over $100,000 when including upstream instrumentation to handle sample preparation. Portable, battery powered, real-time PCR instruments (e.g. Tetracore's T-Core 8, BioFire's Razor EX, and R.A.P.I.D.; Smiths Detection's Bio-Seeq) are available for field use, but are not FDA approved for clinical use. Significant advances are being made in the miniaturization of both real-time PCR and isothermal amplification based diagnostics for use as point of care devices (20-24). Portability and miniaturization are simplified for isothermal amplification methods, making these easier to engineer as "lab-on-a-chip" devices than real-time PCR assays. Further, isothermal methods requiring lower temperatures (e.g., SDA, NASBA, RCA, and RPA) need less power than the high-temperature isothermal technologies, such as LAMP, SmartAmp, and HDA, but these methods generally have reduced sensitivity and require more complex assay design than real-time PCR. Lab-on-a-chip technologies for NAATs are rapidly maturing, with a number of commercial options for point-of-care testing now available, and many more on the horizon. Reviews of commercially available POC diagnostics for detection of infectious disease have been recently published (25-28); however, many of these devices have certain limitations and are not able to serve as the optimal POC device. A recent report from the UC Davis Point-of-Care Technologies Center pointed to deficiencies in the available POC tests for infectious diseases for use in United States disaster caches (29), suggesting the need for further development.

Despite the progress in NAATs, most all FDA-cleared nucleic acid amplification test kits to-date are still categorized as high or moderate complexity under Clinical Laboratory Improvement Amendments (CLIA). Notable exceptions are the Alere™ i Influenza A & B Test, and the Cepheid Xpert Flu+RSV Xpress Test for use on the GeneXpert® Xpress™ System, both of which has been granted waived status under CLIA. High or moderate complexity NAATs may

require nucleic acid preparation from complex sample matrices (e.g. whole blood, stool, etc.), skilled personnel to perform nucleic acid purification and set up of nucleic acid amplification reactions, dedicated instrumentation and laboratory space and qualified personnel to interpret test results. The FilmArray® (BioFire Diagnostics) provides a fully integrated solution including sample preparation, nested RT-PCR followed by multiplex PCR and detection by melt curve analysis (30). Notably, the automated all-in-one device requires little hands-on time and provides an answer in less than 1 hour. Currently FilmArray® Blood Culture Identification (BCID) Panel, Respiratory (RP) Panel, Meningitis Panel (MP), and the Gastrointestinal (GI) Panel are FDA-cleared, and BioFire is pursuing CLIA-waived classification, which would allow the device to be used with minimal training by non-laboratory personnel. While FilmArray® achieves moderate levels of multiplexing of assays (20, 22, 14, and 27 targets in the RP, GI, MP, and BCID Panels respectively), throughput is low, since only one clinical sample can be analyzed per run, and results are qualitative rather than quantitative. In two studies of blood borne infections, the FilmArray® BCID Panel was unable to identify pathogens detected by culture in 8 of 102 cases (31) and in 14 of 167 cases (32). Thus, ~8% of blood borne infections, which are not currently targeted by the FilmArray® BCID Panel will be missed and there is no provision for researchers to add assays to this system. The BCID panel is not a direct specimen test, but provides identification from a positive blood culture, thus pre-culture is required to perform this test. Only pathogens which are targeted by the BCID panel will be identified in this test.

A major limitation of NAATs is the inability to detect an unknown agent that is not targeted by the assays deployed. However, once a novel or emerging agent is identified by next generation sequencing, a NAAT can be designed and used in the laboratory in as little as two weeks

(however, distribution of the test reagents to other laboratories may be subject to FDA regulatory oversight). Another limitation is the ability to multiplex, which is limited to 4-6 targets in a single reaction for real-time PCR, due to the limited number of fluorophores for probes and filters in real-time PCR instruments. Higher levels of multiplexing are readily possible with end-point PCR and post-PCR detection methods. FilmArray®, reviewed above, provides assay multiplexing in the 20-30 target range, but at low throughput. Much higher levels of multiplexing (up to 50-100 targets) is possible with either multiplex ligation-dependent probe amplification (MLPA) (33, 34), multiplexed oligonucleotide ligation-PCR (MOL-PCR) (35) or end-point PCR in combination with Luminex xMAP beads. MLPA and MOL-PCR achieve high levels of multiplexing in a ligation reaction rather than a PCR and the ligation products are subsequently amplified in a single universally primed PCR reaction. The specificity of the ligation reaction in MLPA and MOL-PCR can be applied toward multiple types of genetic markers, including unique sequences, indels, variable repeats or single nucleotide polymorphisms (SNPs) in a single multiplex reaction. Multiplexed readout of PCR products following MLPA, MOL-PCR, or conventional PCR is possible by using capture tags in combination with xMAP beads on the Luminex platform, and this can be performed at high throughput (96 samples per run) when using a liquid handling robot for DNA prep and reaction set-up, providing results in about 5 hours. Currently, xTAG® Gastrointestinal Pathogen and xTAG® Respiratory Viral Panels are available from Luminex for clinical use, providing multiplexed detection for 15 and 12 pathogens respectively. The xTAG procedure involves nucleic acid extraction, multiplex PCR and reverse transcriptase PCR, hybridization to the bead array, and detection on a Luminex or Magpix instrument. Test sensitivity and specificity is between 90 and 100% depending on the pathogen (36). The main advantages of Luminex tests

are the capability for high sample throughput with multiplexed detection, and the ability to create custom designed assays with xMAP beads. However, this system is not an integrated platform, and disadvantages include reduced sensitivity, increased risk of amplicon contamination, less quantitative results, and longer time to results in comparison to real-time PCR. Assay specificity can also be reduced since capture probes may not contribute as much additional specificity as hydrolysis probes.

Microarray-based detection offers another format for multiplexed NAATs. Amplified targets or genomes are detected by hybridization to solid-based or liquid bead-based microarrays. Shorter probes (<25 nucleotides), used in re-sequencing arrays are designed discriminate between pathogens and closely related species and are limited to detecting and differentiating among known agents (e.g. 37, 38). Longer probes (60-70 nucleotides) used in other arrays are able to tolerate sequence mismatches and as such are able to detect novel agents that are similar to known pathogens represented in these arrays (39-42). Arrays can accommodate tens of thousands of probes to hundreds of thousands of probes depending on the array technology used and have been designed to be encompass all viral pathogens, all bacterial pathogens up to pan-microbial arrays that are comprehensive for all known viruses and bacteria (42). Random amplification strategies are used for these arrays and thus host DNA or RNA can be a confounder. Methods of depleting host nucleic acids described in the Next Generation Sequencing section are applicable to enhancing sensitivity for pathogen identification and discovery using microarrays, but this adds further complexity and cost. Microarray-based detection is also poorly suited for complex metagenomic samples. Microarray based methods are not rapid, requiring up to 16 hours for hybridization alone, and 2-3 days for results. Table 1 summarizes many of the important features for NAATs.

Mass Spectrometry Based Assays

Mass spectrometry (MS) provides highly accurate and sensitive analysis of various biomolecules including proteins, lipids, carbohydrates and nucleic acids. MS offers a wide dynamic range in addition to medium- to high-throughput capabilities. Two mass spectrometry technologies are rapidly becoming adopted in clinical diagnostics: matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS and electrospray ionization (ESI) MS. Protein-based MS technologies offer a complementary approach to NAATs by providing detailed analysis of the protein content of complex samples. Two MALDI-TOF-MS systems (BioMérieux's VITEK MS and Bruker's MALDI Biotyper) are currently FDA approved for the identification of bacteria and yeast. Identification is based on matching the measured spectra of protein and peptide molecular weights to a reference database of spectra from known organisms. Advantages over phenotypic, culture-based identification include: rapid identification in minutes, ease-of-use and reduction in hands-on time, low cost per sample, high throughput and sensitivity. These positive features have led to the expanded use of MALDI-TOF-MS for bacterial identification in many clinical laboratories. Microorganism identification by MALDI-TOF-MS still requires a culture of the organism, which remains the rate-limiting step. The accuracy of MALDI-TOF-MS systems for bacterial identification is dependent on the databases they utilize. Gaps in commercial databases can lead to misidentifications (e.g. 43, 44), highlighting the importance of well curated databases, particularly for distinguishing biothreats from their close neighbors. A comparative study of MALDI-TOF-MS with automated microbial growth and detection technology (VITEK 2) found a slightly lower error rate at both the genus and species level of identification with MALDI-TOF-MS (45). However, identification of strain differences within a species by MALDI-TOF-MS remains a challenge.

PCR in combination with ESI-MS can identify bacteria, fungi, viruses, and protozoa using the mass-to-charge ratio of the PCR amplicon to determine its base composition (in conjunction with a database of known organisms). Assays use broad-range primers that target viral, bacterial, and fungal pathogens, with reverse-transcription PCR used for RNA viruses. Unlike NAATs, PCR/ESI-MS uses the nucleic acid amplification only for target amplification, with a separate detection step by ESI-MS. The recently available Abbott PLEX-ID (based on the initial prototype Ibis T5000) PCR/ESI-MS provides additional utility for epidemiological surveillance and environmental biosurveillance over MALDI-TOF-MS systems by directly detecting amplified nucleic acids from complex matrices, such as clinical specimens, food matrices and environmental samples (46, reviewed in 47). The PLEX-ID system has the potential to identify emergent pathogens, in cases where PCR primers amplify a new pathogen strain, and a novel mass is detected. This was demonstrated in the initial identification of influenza virus in the first reported cases of the pandemic 2009 H1N1 pandemic (48). While the time needed for culturing microorganisms is eliminated with the PLEX-ID system, microbial identification requires 6 to 8 hours, depending on the nucleic acid type. An added benefit of PCR/ESI-MS is the ability to detect the presence of specific antibiotic resistance genes contained in the genomes of pathogens of interest. However, the presence of an antibiotic resistance gene does not always equate to phenotypic resistance. PLEX-ID Assays are multiplexed using between 8 PCR primer pairs for the food–borne bacteria kit to 36 PCR primer pairs for the biothreat detection kit.

The limit of detection for PCR/ESI-MS is largely dependent on the PCR amplification step and as such should be similar to that of other multiplex PCR assays. Clinical sensitivity of PCR/ESI-MS has been observed to vary with specimen type. Sensitivity has been reported to be about 50% of the culture-based sensitivity when using 1-ml whole-blood specimens, but a new

integrated specimen preparation technology has been developed that improves the sensitivity to 83% of that of culture (49). The sensitivities for spiked biothreat DNA in bronchoalveolar lavage specimens, compared to standard clinical methods, was 98.5%, while the sensitivity for bacterial, viral and fungal pathogens was 81.8%, 93.3%, and 42.6%, respectively (50). The limit of detection (LOD) for PCR/ESI-MS assays specific for biothreat agents has been estimated by using serial dilutions of purified threat agent nucleic acids, and found to be between 7 and 250 genome equivalents (GE) per well, with most organisms detected at between 15 and 62.5 GE/well (48). The LOD for threat agents spiked into air filter nucleic acid extracts ranged from 40 to 1000 GE/well, with 37.5% (6/16) of the threat agents having LODs of 40 GE/well, 50% (8/16) with LODs of 200 GE/well, and 12.5% (2/16) with LODs of 1000 GE/well (51). At these LODs, the false negative rates were less than 5% for 14 of 16 threat agents and less than 10% for two of 16 agents (51).

Specificities of PCR/ESI-MS versus standard culture-based clinical microbiology methods in bronchial-alveolar lavage specimens has been reported as follows: for spiked biothreat agent DNA 100% specificity; for bacterial pathogens 73.6% specificity; for viral pathogens 97.3% specificity; for fungal pathogens 97.8% specificity (50). In the Sampath *et al*. study, the false positive rates for 15 of 16 threat agents tested was 0%, except for *Rickettsia prowazekii*, which was 14% and was attributed to the presence of near neighbor signatures in the environmental matrix used as a background (51). Similar to MALDI-TOF/MS systems, the accuracy of PCR/ESI-MS systems is dependent on the quality and comprehensiveness of available reference databases.

The Plex-ID system has recently been discontinued by Abbott and is being replaced with the more compact IRIDICA system, which is currently CE Marked (Conformité Européenne) for in vitro diagnostic use in Europe. Similar assays are carried over from the Plex-ID system. A significant criticism of Plex-ID system was its six-figure cost, substantial yearly maintenance expenses and down time associated with equipment failure. Another potential disadvantage for PCR/ESI-MS is the possibility of PCR contamination, and thus strict adherence to segregation of pre- and post-PCR processes and workflows is critical.

While the applications summarized above have focused on pathogen identification, mass spectrometry is also particularly well suited for the detection of biological toxins (52). MS can identify protein-based toxins by their molecular mass, amino acid sequence (including posttranslational modifications), and enzymatic activity (53). Enzymatic activity is determined by measuring the concentrations of the substrate and the resulting cleavage products, which can be done quantitatively by either MALDI-TOF/MS or ESI-MS. The Endopep-MS assay developed by the Centers for Disease Control and Prevention (CDC) for detection and differentiation of the endoproteinase activities of botulinum neurotoxins (BoNT) A-G uses either LC-ESI-MS or MALDI-TOF-MS to detect the synthetic peptides mimicking the target proteins SNAP-25 and VAMP-2, and their cleavage products formed after incubation with BoNT/A-G (54). The incorporation of an antibody affinity method for purification and concentration of BoNT/A, /B, /E, and /F from serum and stool significantly improves the sensitivity of the Endopep-MS assay for use with complex clinical samples (55). The Endopep-MS assay provides excellent sensitivity and specificity, detecting only biologically active toxin. Another major advantage of BoNT Endopep-MS assay is speed: results are provided in hours, compared to the gold standard mouse bioassay, which takes days. Following this same strategy, activity

based MS assays has also been developed for detection of *Bacillus anthracis* lethal factor (LF) (56), *B. anthracis* lethal toxin (LTx) (57) and ricin toxin (58, 59).

The use of antibodies for toxin capture greatly facilitates protein identification by tryptic digestion and amino acid sequencing. This approach has facilitated subtype identification of BoNT/A (60) and has been applied in the forensic identification of ricin (61, 62). A summary of many of the important features of mass spectrometry based assays is presented in Table 2.

Immunological Assays

Immunological assays are widely used in the diagnosis of infectious disease and for the identification of potential biothreat and infectious disease agents. Immunological assays with defined antibodies can be used to detect bacterial cells, spores, viruses and toxins, while serological tests are used to monitor the immune response to such agents by detecting and measuring circulating antibodies recognizing these agents. Conventional serology can be performed using variety of techniques including enzyme-linked immunosorbent assays (ELISA), agglutination, precipitation, complement-fixation, and fluorescent antibodies—supporting both direct and indirect fluorescent antibody tests. Serology is important for epidemiological studies when exposure without development of disease is an important parameter in the spread and control of disease. Serology provides an indirect diagnosis of infectious disease by measuring the humoral immune response, which is typically polyclonal and varies among individuals depending on their genetic background, their prior history of exposure to infectious agents and the time interval since the most recent infection (63). A further complexity can arise from antigenic variation of an infectious agent, which can lead to different serotypes. Therefore,

reactivity of patient serum samples in serological tests may not be precise, predictable or definitive.

Automated systems that are compatible with a variety of commercially available assays are available for agglutination tests (e.g., OC Sensor series, Eiken Chemical Co., Japan; FluHema™ Hemagglutination Analyzer, SciRobotics; Cypher One™, InDevR, Boulder and described in (64, 65), complement-fixation tests (Seramat system, Diesse, Monteriggioni, Italy) and ELISA (JANUS, PerkinElmer; EL406, BioTek; Agility®, Dynex; ThunderBolt®, Gold Standard Diagnostics).

ELISA provides a format that is not only amenable to high throughput automation but can be formatted to detect either antibodies produced during an immune response or the pathogen. Other commonly used formats for immunoassays include:, lateral flow immunoassays (LFA), time-resolved fluorescence (TRF) assays, and immunomagnetic separation-electrochemiluminescence (IMS-ECL) assays (66).

ELISAs for infectious agents have had a dramatic impact on disease diagnosis by simplifying detection and shortening the time required to reach conclusive results to 2-4 hrs, compared to days for culture-based methods. ELISAs are versatile, robust, economical and relatively simple to perform. Having the capture antibody immobilized to a solid surface facilitates separation of bound from non-bound material during the assay. This ability to wash away nonspecifically bound materials makes the ELISA a powerful tool for measuring specific analytes within complex or crude preparations. Specificity and sensitivity of ELISAs, and other formats of immunological assays, are significantly enhanced by the use of capture and detector antibodies that recognize orthogonal epitopes allowing simultaneous binding to the antigen. The use of two

antibodies to provide detection signals in a single assay significantly reduces background by effectively reducing false positives due to non-specific binding. Monoclonal antibodies can further increase specificity and reduce background, while polyclonal antibodies can increase coverage to detect a broader range of isolates belonging to a given species and usually have higher affinity. Combinations of monoclonal and polyclonal antibodies can be used to balance sensitivity and specificity. High throughput ELISAs can also be performed, using microtiter plates or microarray formats (67), although no more than ~35 microarray sandwich assays can be multiplexed simultaneously in a single volume, as interfering cross talk between different capture and detection antibodies may occur above this level.

ELISAs can be configured to either detect antibodies in the case of antibody-capture ELISA (antigen is immobilized) or antigens in antigen-capture ELISA (antibody is immobilized). Antibody-capture ELISAs are useful in the diagnosis of several infectious diseases (e.g. rubella, measles, toxoplasmosis, Lyme disease, HIV, dengue and West Nile virus). Antibody-capture ELISAs are particularly sensitive for detecting IgM responses early in an infectious disease. Antibody-capture ELISAs can be antibody isotype specific, involving affinity purification of the specific immunoglobulin isotypes (i.e. IgM, IgG or IgA) from the patient specimen, followed by detection of each antibody isotype to the specific infectious agent. The IgM/IgG ratio obtained from antibody capture ELISAs can be very useful in distinguishing primary from secondary dengue virus infections (68). Antigen-capture ELISAs are useful in detecting acute infection (e.g. avian influenza, Ebola, salmonella, dengue NS1, and amoebic colitis), and can often be used earlier, as the pathogen itself is detected, and there is no need to wait for the development of specific antibodies.

One of the factors which may limit the use of ELISA assays is the need to coat plates with capture antibodies before use and the requisite incubation time needed for detection. Pre-coated plates are available for some assays but have a limited shelf life, which could result in additional costs associated from replacing expired stock.

Lateral flow immunoassays (LFAs) are among the simplest to perform, with results in 15-30 minutes. Such handheld assay devices are well suited for point of care testing and field use. LFAs are typically performed on nitrocellulose or cellulose acetate membranes using sandwich-type assays, with gold nanoparticle labeled antibodies used for the colorimetric readout. Reliability of test results may be improved with the addition of reader device. Multiplexing at modest levels in LFAs has been described for botulinum neurotoxin serotype-A and –B (69), three serogroups of Shiga toxin-producing *Escherichia coli* (70), and for the viral bloodborne pathogens HIV, HCV, and HAV (71). Tetracore and InBios have a series of biological threat agent kits available which combine lateral flow immunoassay test strips with a handheld reader device. Singleplex LFAs are currently available for abrin toxin, ricin toxin, botulinum toxin A, SEB, *Bacillus anthracis*, *Brucella* sp, *Yersinia pestis*, *Francisella tularensis* and Orthopox viruses from Tetracore and *Burkholderia mallei*, *Burkholderia pseudomallei*, and Dengue fever virus from InBios (72). However, recent examination and comparison of immunological field tests for ricin (73) or bolutinum toxin (74) show that most of these commercial assays perform very poorly. However recent evaluations of LFAs for ricin and abrin have found these to be both sensitive and specific (75, 76). LFAs have the convenience of portability and speed (15-30 minutes to result) but usually have reduced sensitivity compared to NAAT or ELISA tests. LFAs are designed for individual tests, not for high-throughput screening and results are

qualitative or at best semi-quantitative. Also regulatory agencies often require that independent testing be performed on samples that test positive with a LFA test.

Despite evidence of modest sensitivity in medical settings, rapid antigen tests are available to clinics and the public for POC or home testing. Rapid antigen tests for influenza A and B, respiratory syncytial virus, and group A streptococcus are among the most widely used (77-79). Sensitivity of these tests varies depending on the target analyte, timing of testing after onset of symptoms, and other factors including skill of the person performing the test (79, 80).

Time-resolved fluorescence (TRF) assays are designed to detect the presence of a biomolecule using lanthanide chelate labeled reagents and separating the unbound reagent using wash steps (e.g. 81). TRF assays are flexible, compatible with a variety of plate readers, and employ a wash-based technology that remains compatible with most sample types. The fluorescence decay time of lanthanide chelate labels is much longer than traditional fluorophores, allowing efficient use of temporal resolution for reduction of auto-fluorescent background. A large Stokes' shift between excitation and emission wavelengths and the narrow emission peaks contribute to improved signal-to-noise ratio. TRF assays are well suited for clinical immunoassays but have limitations with environmental samples that contain naturally occurring lanthanides and which result in increased background and reduced sensitivity.

Immunomagnetic separation-electrochemiluminescence (IMS-ECL) assays combine immunomagnetic separation with electrochemiluminescence detection. This method has been applied toward the detection of *E. coli* O157 and *Salmonella typhimurium* in foods with detection limits of 10^2-10^3 bacteria/ml of food sample (82) and of *B. anthracis* spores in soil with a detection limit of 10^2 to 10^5 spores in buffer (83). Total processing time for IMS-ECL assays

is 1-1.5 h. A summary of many of the important features of immunological-based assays is presented in Table 3.

Next-Generation Sequencing

Advances in next-generation sequencing (NGS) have fueled the exponential growth of high quality draft and complete genome sequences of bacterial, viral and fungal pathogens and also thousands of non-pathogenic species representing much of the phylogenetic diversity of bacteria and archaea (84, 85). Generation of more complete genome datasets for pathogens and their near neighbors in turn, leads to improved nucleic acid-based diagnostics, which are designed with an improved representation and understanding of the targeted genome diversity and the non-targeted neighbor genomes. The massive-throughput made available by NGS has spurred the development and application of metagenomic shotgun sequencing to community genomics, which focuses on elucidating the genomic content of microbial communities in complex environments such as soils (86) and the human gut (87). The development of these capabilities and approaches laid the groundwork for the Human Microbiome Project (88), providing significant insights into the function and diversity of the healthy human microbiome (89, 90). Recently there has been great interest in applying metagenomic shotgun sequencing of DNA or RNA from patient samples to detect the full range of infectious agents: from bacteria and viruses to eukaryotic pathogens (91).

Next-generation sequencing can fill an important gap in identifying the etiological agent of an infectious disease in cases where existing diagnostics fail. In particular, NGS can identify new or emerging agents for which diagnostics are not available, as in the cases of Bas-Congo rhabdovirus (92), MERS (Middle East Respiratory Syndrome) coronavirus (93), Lujo virus [a

new hemorrhagic fever-associated arenavirus from southern Africa (94)], a novel polyomavirus in human Merkel cell carcinoma (95), and novel neuroinvasive astroviruses causing encephalitis in two immunocompromised patients (96, 97). As early as 2003, random PCR and a degenerative coronavirus primer strategy were used to amplify fragments of a viral genome, with subsequent sequencing resulting in the identification of the novel SARS coronavirus in patients with a severe acute respiratory disease (98, 99). Viral metagenomics has also been applied in several relevant settings, including: a public health enterovirus surveillance program (to investigate unidentified viruses in cell cultures from clinical isolates where standard PCR assays failed to detect viruses) (100), in a veterinary diagnostic laboratory (to identify viral etiological agents directly from clinical specimens without culturing) (101), and in a virology research laboratory (to identify novel viruses in homogenized tissues of acutely infected mice) (102). These studies demonstrate that random nucleic acid amplification followed by unbiased next-generation sequencing directly from complex samples, including clinical specimens, is an effective strategy to identify novel pathogens, especially viruses. The identification of novel or emerging pathogens by NGS of clinical samples can provide a correlation with a disease but fails to fulfill Koch's postulates for disease causation. However when NGS is applied toward appropriate controlled sets of samples, and the data is interpreted with a current understanding of the biology of the pathogen, establishing whether a correlation is causal or opportunistic can become more scientifically based (103, 104). Great care should always be exercised in eliminating or accounting for possible contaminating nucleic acids, which can arise from laboratory reagents (105-109).

The choice of NGS platform and conditions affecting read length and depth of sequencing are important parameters in pursuing an unbiased next-generation sequencing strategy. There are

also methods for pathogen enrichment or host depletion that can increase sensitivity for identification of novel agents in clinical samples. Relatively simple viral particle purification procedures include: repeated freeze/thaw cycles to release virus from infected cells, low speed centrifugation to pellet cellular debris followed by ultrafiltration, and high speed centrifugation to concentrate virus particles. More specific purification methods may be used, including affinity chromatography or density gradient centrifugation. Viral capsid and viral envelope protect viral nucleic acid from nuclease digestion and thus nuclease digestion before purification of viral nucleic acids can significantly enrich for viral nucleic acid by eliminating much of the host DNA and/or RNA (110). The effectiveness of these rapid and simple techniques for the enrichment of viruses prior to metagenomic sequencing has been recently published (111, 112). Additional strategies to deplete background host nucleic acids can also be implemented, including the use of methylation-specific restriction endonucleases to selectively degrade host DNA (113), the use of methyl-CpG binding domain antibodies to separate methylated host DNA from microbial DNA based on differential CpG methylation density (114), the use of C_0t reassociated DNA and double strand specific nucleases to remove abundant human DNA (115), and the removal of host ribosomal RNA by subtractive hybridization and exonuclease digestion (116). Methods to reduce host DNA add more labor, costs and complexity to the process however.

Target enrichment strategies can also be applied to metagenomic approaches when looking for known or closely related pathogens. These methods were originally developed for selective re-sequencing of the human exome or collections of genes involved in cancer (117). Target enrichment can be achieved by targeted amplification methods involving multiplexed PCR or highly parallel microdroplet singleplex PCR or molecular inversion probes and by hybrid

capture, either on arrays or in solution (113). A whole genome hybrid capture method has been shown to effectively enrich for *Plasmodium vivax* DNA from contaminating human DNA for more efficient whole genome sequencing and analysis (118, 119). Solution hybrid capture has also been used for the enrichment of *Borrelia burgdorferi* DNA from an arthropod vector (120). Given the large target size of up to 200 Mb that can be captured with current technology it should be relatively straightforward to design broad range capture kits designed to cover genomic regions providing diagnostic value for a wide range pathogens responsible for bacteremia, gastrointestinal infections, pulmonary infections, etc. But such an approach would be take more time, require more labor and be more expensive than detection by real-time PCR.

Even with the use of target enrichment or background depletion approaches, sequence-independent amplification is frequently needed to amplify the enriched or depleted nucleic acid preparations to generate sufficient material for metagenomic sequencing. A variety of methods are available for sequence independent amplification of DNA and RNA including: sequence-independent single primer amplification (SISPA; an adapter-ligated PCR method which has been further developed to amplify single stranded or double stranded DNA or RNA) (121), random PCR [rPCR; developed to make random-primed cDNA libraries from low amounts of RNA (122, 123), and further adapted to randomly amplify DNA (124)], degenerate oligonucleotide-primed PCR (125), and primer extension PCR (126). However, PCR-based methods of random amplification may introduce sequence-dependent bias resulting in uneven coverage of amplified targets (125).

Commercially available whole genome amplification (WGA) kits include REPLI-g (Qiagen) that is based on multiple displacement amplification using phi29 polymerase in an isothermal

amplification reaction (127, 128), and GenomePlex (Sigma-Aldrich) or PicoPLEX (NEB) developed by Rubicon Genomics (129) which uses a proprietary amplification technology based upon random fragmentation of genomic DNA (~1.5Kb) and conversion of the resulting small fragments to PCR-amplifiable molecules flanked by universal priming sites. Experimental comparisons of these two whole genome amplification techniques have been reported (130, 131). A review of how each of the sequence-independent amplification methods has been applied toward the discovery of novel viruses is available (132).

Whole genome sequencing of bacterial isolates can also be used as a strategy to serotype isolates. This approach has been demonstrated by the Gastrointestinal Bacteria Reference Unit at the Public Health England national reference laboratory in the serotyping of 682 *E. coli* strains (133). This group developed SerotypeFinder, a user-friendly Web-based analysis tool for whole-genome sequencing (WGS) data (133). They show that *E. coli* serotyping performed solely from WGS data, provides faster and cheaper typing than current routine procedures and making WGS typing a superior alternative to conventional typing strategies (133).

While metagenomic shotgun sequencing has the potential to detect a wide range of infectious agents, the identification of that agent in a background of human or commensal flora remains a challenging problem. For clinical diagnosis, computational methods to handle the large next-generation sequencing data sets need to be both fast and accurate. A number of computational tools have been developed for classification of metagenomic datasets. These include short read alignment tools (134-137) and sequence composition tools (138-143). Reviews of metagenomic sequence classification tools were published in 2012 (142, 143) and include a comparative evaluation of many of the tools available at that time (143). These comparative evaluations

showed that NBC, a naïve Bayes classification tool (138) exhibited the highest accuracy and sensitivity at the genus level (among 134-136 and 139), but that NBC, as well as other probabilistic methods (135) and BLAST-based methods (134, 136) are computationally expensive.

Several faster methods such as Genometa, which uses the Bowtie aligner (144); RITA, a hybrid classifier based on Discontiguous MEGABLAST and Naïve Bayes (145); KRAKEN, using exact alignment of k-mers (141); Sequedex, using exact matches to a precomputed set of peptide 10-mers (146); GOTTCHA, using exact matches to a unique signature database (147); and WGSQuikr, based on k-mer frequency (148) have been developed but their performance still does not match NBC's sensitivity. Thus there appears to be a trade off in sensitivity versus speed for classification tools. LMAT (140) leverages large single address space memory to efficiently assign taxonomic labels to individual reads in large metagenomic datasets and uses a reduced pathogen library (having removed non-informative regions) that fits in conventional-sized memory. Some tools such as MetaPhlAn (137) and WGSQuikr (148) calculate the proportion of each organism present in the sample based on read classification which may help in determine the state of an infection and contribute toward the diagnosis of co-infections. These and other quantitative metagenomic analysis methods, normalize read counts based on average genome size, which improves accuracy (149-152).

While many of the first tools developed for metagenomic analysis were designed for analysis of bacterial communities, several of the more recent tools such as LMAT (140), Sequedex (146) and GOTTCHA (147) also support detection and classification of viruses. There are also a number of tools available that are designed exclusively for virus detection and discovery in

metagenomic datasets, including those from clinical samples (153-158). Beyond virus identification and discovery, NGS enables studies of viral dynamics during the course of infection that have not previously been possible (159). These viral dynamics studies are particularly relevant to human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV), where many genetically related variants, called quasi-species, evolve over time due to immune pressure and antiviral therapy (160). Evolutionary dynamics of the Ebola virus during the 2014 epidemic in Sierra Leone was revealed by NGS studies (161-163) providing insights into virus evolution during human to human transmission and showing how purifying selection acts at different timescales. NGS also provides an effective tool for the detection of virus integration events in host genomes, which is clinically relevant for a number of viruses (164). For example, integration of HIV at specific genomic locations can lead to clonal expansion and persistence of virus-infected cells under combination antiretroviral therapy (165) and integration of HBV into specific gene targets is associated with the development of HBV-related hepatocellular carcinomas (166).

Despite the promise and proven utility for NGS in clinical microbiology and public health surveillance, its "widespread implementation in clinical and public health microbiology laboratories is limited by the need for effective semi-automated pipelines, standardized quality control and data interpretation, bioinformatics expertise, and infrastructure" (167). The complexity of data analyses on very large NGS data sets, remains a significant barrier for non-bioinformaticians. To meet clinical microbiology and public health needs, NGS data analysis pipelines will need to provide quick and accurate results, a user-friendly interface for laboratory staff, and automatic tracking of samples and data analyses for data provenance and audit purposes (168). Additionally, stringent protocols for sequencing and analysis that mitigate

contamination from environmental sources and between experiments are essential for clinical analysis (169). Good laboratory practices for clinical next-generation sequencing and informatics pipelines have been described (170-172), and cover the production of sequencing reads and assignment of base quality scores, the de-multiplexing of reads, their alignment to a reference sequence and variant calling, but these have been focused on variant calling for clinical genetics and have not adequately addressed the additional complexities associated with metagenomic sequencing for the clinical diagnosis of infectious diseases. The National Institute of Standards and Technology (NIST) recently convened a workshop to identify priority areas for standards activities to facilitate the development of a measurement infrastructure for NGS-based pathogen identification (173). Bioinformatic challenges and solutions associated with bacterial isolate whole genome sequencing (WGS) as a molecular diagnostic have been addressed (167, 174, 175). WGS has contributed significantly to outbreak investigations involving *Clostridium difficile*, enterohemorrhagic *E. coli*, carbapenem-resistant *Klebsiella pneumonia*, Methicillin-resistant *Staphylococcus aureus, Legionella pneumophila* and *Mycobacterium tuberculosis* (reviewed in 174-176). WGS provides the highest resolution of information for strain characterization and epidemiological analyses, and over time can be expected to replace traditional typing methods, resistance gene detection and other sequence-based methods (e.g., MLST, 16S rDNA, etc.). However, while WGS can readily detect acquired antibiotic resistance genes such as beta-lactamases and aminoglycoside modifying enzymes, there remain limitations for predicting resistance mechanisms conferred by mutations in regulatory systems (reviewed in 167). While antimicrobial susceptibility testing cannot currently be replaced with WGS, current limitations will diminish as genomic variants responsible for drug resistance phenotypes are

further compiled. WGS resistance gene profiles have been shown to be clinically relevant for slow growing (e.g. *Mycobacterium tuberculosis*) or difficult to culture organisms (177).

There are additional bioinformatics challenges associated with metagenomic sequencing for clinical diagnosis of infectious disease. Recently a number of groups have developed bioinformatics tools and pipelines attempting to address some of these challenges (178-187). In general, these tools begin with a subtractive phase in which reads aligning to the human genome are removed and then remaining reads are aligned to microbial reference sequences and used to create alignment-based assemblies and unaligned reads are assembled *de novo*. Assembled sequences failing to align to microbial reference sequences can be further analyzed for evidence of being novel viruses or pathogens. Early tools (178-180) largely followed this approach, using fast alignment algorithms for host subtraction, while relying on traditional BLAST for final pathogen determination. Pathoscope (181) omitted the time-consuming assembly step and instead uses a Bayesian statistical framework to match reads to a known database of target genomes. Clinical PathoScope (184) incorporates the original PathoScope algorithm into an improved pipeline incorporating removal of contaminating sequences from the host and commensal microbes for host-dominated clinical samples. CoMPASS (182) provides a dual approach for analysis of pathogen and host meta-transcriptome (RNA-seq) datasets using largely open source programs. The SURPI pipeline for pathogen detection (183) speeds this up further by leveraging the fast alignment tools SNAP and RAPSearch in a fast mode against viral and bacterial databases and in a comprehensive mode against the entire NCBI nt database. Novel pathogens may be identified through the use of more sensitive amino acid alignments to protein databases. The MetaGeniE pipeline (185) generates an all-against-all comparison dataset between the reads and the reference database and then uses these results to generate cumulative

statistics from combined local and global alignments. The GOTTCHA (147) algorithm has been incorporated into a highly adaptable bioinformatics platform called EDGE (187) that allows laboratories to quickly analyze and interpret metagenomic sequence data for a wide range of use cases including clinical samples, and complex environmental samples. The SEAR pipeline (186) is designed for the detection of horizontally acquired antimicrobial resistance genes in metagenomic sequencing data. A summary of many of the important features of next-generation sequencing based assays is presented in Table 4.

Summary

The features, benefits, and limitations of 4 different types of CIDTs for detection and identification of pathogens, including biothreat agents, have been summarized. These include nucleic acid amplification assays, mass spectrometry assays, immunological assays and next generation sequencing. A fundamental feature of CIDTs over traditional culture based methods is that results can generally be obtained more rapidly, which can be critical for clinical decision-making. Other benefits of CIDTs include greater sensitivity than culture (in many circumstances), ease of use (for many of the CIDTs), ability to detect new, emerging or rarely seen pathogens, and a better ability to detect co-infections (188). However, most CIDTs in use today remain narrow in scope and can fail to detect the etiological agent in a significant percentage of cases. In these cases, in particular, unbiased next-generation sequencing holds great promise for comprehensive detection of pathogens from clinical samples. All of these benefits can contribute toward improved pathogen and disease surveillance. Current limitations of most CIDTs include: 1) an inability to distinguish live from dead organisms, which may be important in characterizing the state of disease in a patient or the threat posed by an infectious

agent in food or other matrix; and 2) limited ability to access antimicrobial resistance and other microbial characteristics.

Culture-based diagnostics remain widely in use to support diagnosis and characterization of bacterial and fungal pathogens causing bacteremia, fungemia, meningitis and food borne diseases, in part due to some of the current limitations of CIDTs and in part to fulfill case reporting requirements. Culture-based diagnostics have also advanced with a number of automated microbiology growth and detection systems available (e.g., VITEK 2 (bioMérieux), MicroScan (Siemens Healthcare), Phoenix and BACTEC (Becton Dickinson)) to support pathogen identification and characterization.

The four CIDT technologies reviewed here are complementary and augment traditional culture-based diagnostics. Large clinical diagnostic laboratories will have use for all four of these technologies. POC diagnostic tests are best represented by NAATs and various formats of immunological assays. Syndromic formats of POC tests in which assays simultaneously detect the most common etiological agents of a given syndrome are becoming increasingly available and offer greater opportunity to identify the cause of disease in POC settings (189).

For biosurveillance, NAATs in general, and real-time PCR specifically, offers the widest application space in terms of sample matrices (clinical, food, water, environmental) due to their specificity in complex matrices. NAATs also tend to be more sensitive than antibody-based detection methods, with real-time PCR assays being able to detect 10 or fewer microorganisms in a little as 30 minutes. The limitation or rate-limiting step is the need for sample preparation to isolate and purify nucleic acids. The other limitation is the ability to multiplex, which is limited to 4-6 targets at the current time for real-time PCR. Much higher levels of multiplexing is

possible with end point PCR methods using the Luminex system, but sensitivity, quantitative dynamic range and specificity is reduced. The availability of fully integrated systems, incorporating sample processing prior to NAATs with high multiplexing capability, providing quantitative information with high sensitivity and specificity is lacking and remains an opportunity for further commercial development. The Cepheid GeneXpert® System, which fully integrates and automates sample extraction, amplification, and detection in a single cartridge is perhaps the most advanced system to date, but remains limited to multiplexing of 6 assays.

Immunological assays are generally quick and convenient and are used in a wide variety of tests, but are only applicable for known pathogens or antibodies. To detect a particular antibody or antigen, a known reciprocal antigen of antibody must be generated. Quality of assays is highly reagent specific with nonspecific binding of poor quality antibody or antigen leading to false positive results or high backgrounds. Detection by immunological assays is limited by the affinity and specificity of the antibodies used and the abundance of the target antigen. Also, antibodies can have limited stability affecting shelf-life. Until recently, antibodies were generated by traditional immunization and B cell immortalization by fusion to partner myeloma cell lines to create hybridomas producing monoclonals. However, recent advances in the generation of monoclonal antibodies using in vitro methods of phage and yeast display (190, 191) has reduced the time and expense of generating specific reagents. Furthermore the availability of the sequences of selected recombinant clones enables the use of in vitro evolution approaches to improve stability, specificity and/or affinity of respective heavy and light chain genes, which may lead to better performance in this area in the future. It has been recently been proposed (192, 193) that the use of antibody sequences as barcodes to unequivocally identify antibodies used in research or diagnostics, will go a long way toward improving reproducibility.

Mass spectrometry based technologies have seen an increased role in laboratory-based diagnostics by automating identification of bacteria and yeast with commercial systems and are also ideally suited for identification of biologically active toxins. However, instrument cost and service contracts remain high, limiting their use to large diagnostic laboratories.

Next-generation sequencing fills a vital role in characterization of disease outbreaks by whole genome sequencing of isolates and in the identification of infectious agents when other diagnostics fail, particularly for rare, emerging and novel pathogens. New sequence of novel and emerging pathogens enhance GenBank and support the development of new NAATs for detection and diagnosis. NGS is also well suited for forensics. Instrument costs are significant however and data interpretation can be complex. Currently NGS is not as well suited for environmental biosurveillance for specific pathogens compared to real-time PCR due to the complexities of metagenomic sequence analysis and the cost of metagenomic sequencing.

It is clear from this review that despite the many recent advances in culture-independent diagnostics that a single technology does not yet exist that can meet the majority needs of both clinical diagnostics and environmental biosurveillance. Many CIDTs are well suited for particular diagnostic or detection questions and this review may help direct the reader to these best use application spaces. Also as CIDTs continue to mature, it is likely that some of these could be plugged early warning systems for disease surveillance, by incorporating wireless reporting of results through smart phones to online surveillance systems. Such a scenario would be applicable to both the developed world and developing nations. What would an ideal platform for clinical diagnostics and environmental biosurveillance look like? Some features may be unique to biosurveillance and others will be useful for most clinical diagnostic situations.

Ideally a platform should be portable, easy to use, and capable of detecting multiple agents simultaneously. Platforms that integrate sample processing will have the benefit of reduced complexity for the operator. The sample processing method should be applicable for all sample types and all target analytes. Assays employed by the platform should be: configurable for both clinical and select agent detection, sensitive, specific, and capable of detecting low concentrations of target agent without interference from diverse background materials (reviewed in 194). For the platform to be commercially viable it would need to be affordable and easily utilized in biothreat situations, national emergencies, or naturally occurring epidemics, *in addition* to routine clinical diagnostic use.

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References

1. Monis PT, Giglio S. Nucleic acid amplification-based techniques for pathogen detection and identification. Infect Genet Evol. 2006;6(1):2-12.
2. Mothershed EA, Whitney AM. Nucleic acid-based methods for the detection of bacterial pathogens: present and future considerations for the clinical laboratory. Clin Chim Acta. 2006;363(1-2):206-20.
3. Gill P, Ghaemi A. Nucleic acid isothermal amplification technologies: a review. Nucleosides Nucleotides Nucleic Acids. 2008;27(3):224-43.
4. Craw P, Balachandran W. Isothermal nucleic acid amplification technologies for point-of-care diagnostics: a critical review. Lab Chip. 2012;12(14):2469-86.
5. Chang CC, Chen CC, Wei SC, Lu HH, Liang YH, Lin CW. Diagnostic devices for isothermal nucleic acid amplification. Sensors (Basel). 2012;12(6):8319-37.
6. Li J, Macdonald J. Advances in isothermal amplification: novel strategies inspired by biological processes. Biosens Bioelectron. 2015;64:196-211.
7. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, Bright IJ, Lucero MY, Hiddessen AL, Legler TC, Kitano TK, Hodel MR, Petersen JF, Wyatt PW, Steenblock ER, Shah PH, Bousse LJ, Troup CB, Mellen JC, Wittmann DK, Erndt NG, Cauley TH, Koehler RT, So AP, Dube S, Rose KA, Montesclaros L, Wang S, Stumbo DP, Hodges SP, Romine S, Milanovich FP, White HE, Regan JF, Karlin-Neumann GA, Hindson CM, Saxonov S, Colston BW. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem. 2011;83(22):8604-10.
8. Centers for Disease Control and Prevention. Recommendations for the laboratory-based detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*--2014. MMWR Recomm

Rep. 2014;63(RR-02):1-19.

9. van Elden LJ, van Kraaij MG, Nijhuis M, Hendriksen KA, Dekker AW, Rozenberg-Arska M, van Loon AM. Polymerase chain reaction is more sensitive than viral culture and antigen testing for the detection of respiratory viruses in adults with hematological cancer and pneumonia. Clin Infect Dis. 2002;34(2):177-83.
10. Loens K, Goossens H, de Laat C, Foolen H, Oudshoorn P, Pattyn S, Sillekens P, Ieven M. Detection of rhinoviruses by tissue culture and two independent amplification techniques, nucleic acid sequence-based amplification and reverse transcription-PCR, in children with acute respiratory infections during a winter season. J Clin Microbiol. 2006;44(1):166-71.
11. Honoré-Bouakline S, Vincensini JP, Giacuzzo V, Lagrange PH, Herrmann JL. Rapid diagnosis of extrapulmonary tuberculosis by PCR: impact of sample preparation and DNA extraction. J Clin Microbiol. 2003;41(6):2323-9.
12. Rovery C, Greub G, Lepidi H, Casalta JP, Habib G, Collart F, Raoult D. PCR detection of bacteria on cardiac valves of patients with treated bacterial endocarditis. J Clin Microbiol. 2005;43(1):163-7.
13. Birch L, Dawson CE, Cornett JH, Keer JT. A comparison of nucleic acid amplification techniques for the assessment of bacterial viability. Lett Appl Microbiol. 2001;33(4):296-301.
14. Keer JT, Birch L. Molecular methods for the assessment of bacterial viability. J Microbiol Methods. 2003;53(2):175-83.
15. Cangelosi GA, Meschke JS. Dead or alive: molecular assessment of microbial viability. Appl Environ Microbiol. 2014;80(19):5884-91.
16. Institute of Medicine and National Research Council. *BioWatch PCR Assays: Building*

Confidence, Ensuring Reliability; Abbreviated Version. Washington, DC: The National Academies Press, 2015.

17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. Methods. 2001;25(4):402-8.
18. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29(9):e45.
19. Popowitch EB, O'Neill SS, Miller MB. Comparison of the Biofire FilmArray RP, Genmark eSensor RVP, Luminex xTAG RVPv1, and Luminex xTAG RVP fast multiplex assays for detection of respiratory viruses. J Clin Microbiol. 2013;51(5):1528-33.
20. Almassian DR, Cockrell LM, Nelson WM. Portable nucleic acid thermocyclers. Chem Soc Rev. 2013;42(22):8769-98.
21. Ahmad F, Hashsham SA. Miniaturized nucleic acid amplification systems for rapid and point-of-care diagnostics: a review. Anal Chim Acta. 2012;733:1-15.
22. Asiello PJ, Baeumner AJ. Miniaturized isothermal nucleic acid amplification, a review. Lab Chip. 2011;11(8):1420-30.
23. Park S, Zhang Y, Lin S, Wang TH, Yang S. Advances in microfluidic PCR for point-of-care infectious disease diagnostics. Biotechnol Adv. 2011;29(6):830-9.
24. Craw P, Balachandran W. Isothermal nucleic acid amplification technologies for point-of-care diagnostics: a critical review. Lab Chip. 2012;12(14):2469-86.
25. Holland CA, Kiechle FL. Point-of-care molecular diagnostic systems--past, present and future. Curr Opin Microbiol. 2005;8(5):504-9.
26. Lebovitz EE, Burbelo PD. Commercial multiplex technologies for the microbiological diagnosis of sepsis. Mol Diagn Ther. 2013;17(4):221-31.

27. Howick J, Cals JW, Jones C, Price CP, Plüddemann A, Heneghan C, Berger MY, Buntinx F, Hickner J, Pace W, Badrick T, Van den Bruel A, Laurence C, van Weert HC, van Severen E, Parrella A, Thompson M. Current and future use of point-of-care tests in primary care: an international survey in Australia, Belgium, The Netherlands, the UK and the USA. BMJ Open. 2014;4(8):e005611.

28. Reddington K, Tuite N, Minogue E, Barry T. A current overview of commercially available nucleic acid diagnostics approaches to detect and identify human gastroenteritis pathogens, Biomol Detect Quantif. 2014;1(1): 3-7.

29. Curtis CM, Louie RF, Vy JH, Ferguson WJ, Lam M, Truong AT, Rust MJ, Kost GJ. Innovations in point-of-care testing for enhanced United States disaster caches. Am J Disaster Med. 2013;8(3):181-204.

30. Poritz MA, Blaschke AJ, Byington CL, Meyers L, Nilsson K, Jones DE, Thatcher SA, Robbins T, Lingenfelter B, Amiott E, Herbener A, Daly J, Dobrowolski SF, Teng DH, Ririe KM. FilmArray, an automated nested multiplex PCR system for multi-pathogen detection: development and application to respiratory tract infection. PLoS One. 2011;6(10):e26047.

31. Blaschke AJ, Heyrend C, Byington CL, Fisher MA, Barker E, Garrone NF, Thatcher SA, Pavia AT, Barney T, Alger GD, Daly JA, Ririe KM, Ota I, Poritz MA. Rapid identification of pathogens from positive blood cultures by multiplex polymerase chain reaction using the FilmArray system. Diagn Microbiol Infect Dis. 2012;74(4):349-55.

32. Altun O, Almuhayawi M, Ullberg M, Ozenci V. Clinical evaluation of the FilmArray blood culture identification panel in identification of bacteria and yeasts from positive blood culture bottles. J Clin Microbiol. 2013;51(12):4130-6.

33. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res. 2002;30(12):e57.

34. Bergval I, Sengstake S, Brankova N, Levterova V, Abadía E, Tadumaze N, Bablishvili N, Akhalaia M, Tuin K, Schuitema A, Panaiotov S, Bachiyska E, Kantardjiev T, de Zwaan R, Schürch A, van Soolingen D, van 't Hoog A, Cobelens F, Aspindzelashvili R, Sola C, Klatser P, Anthony R. Combined species identification, genotyping, and drug resistance detection of Mycobacterium tuberculosis cultures by MLPA on a bead-based array. PLoS One. 2012;7(8):e43240.

35. Deshpande A, Gans J, Graves SW, Green L, Taylor L, Kim HB, Kunde YA, Leonard PM, Li PE, Mark J, Song J, Vuyisich M, White PS. A rapid multiplex assay for nucleic acid-based diagnostics. J Microbiol Methods. 2010;80(2):155-63

36. Reddington K, Tuite N, Minogue E, Barry T. A current overview of commercially available nucleic acid diagnostics approaches to detect and identify human gastroenteritis pathogens. Biomol Detect Quant, 2014;1(1):3-7.

37. Lin B, Blaney KM, Malanoski AP, Ligler AG, Schnur JM, Metzgar D, Russell KL, Stenger DA. Using a resequencing microarray as a multiple respiratory pathogen detection assay. J Clin Microbiol. 2007;45(2):443-52.

38. Wong CW, Albert TJ, Vega VB, Norton JE, Cutler DJ, Richmond TA, Stanton LW, Liu ET, Miller LD. Tracking the evolution of the SARS coronavirus using high-throughput, high-density resequencing arrays. Genome Res. 2004;14(3):398-405.

39. Wang D, Coscoy L, Zylberberg M, Avila PC, Boushey HA, Ganem D, DeRisi JL. Microarray-based detection and genotyping of viral pathogens. Proc Natl Acad Sci U S A.

2002;99(24):15687-92.

40. Chou CC, Lee TT, Chen CH, Hsiao HY, Lin YL, Ho MS, Yang PC, Peck K. Design of microarray probes for virus identification and detection of emerging viruses at the genus level. BMC Bioinformatics. 2006;7:232.

41. Palacios G, Quan PL, Jabado OJ, Conlan S, Hirschberg DL, Liu Y, Zhai J, Renwick N, Hui J, Hegyi H, Grolla A, Strong JE, Towner JS, Geisbert TW, Jahrling PB, Büchen-Osmond C, Ellerbrok H, Sanchez-Seco MP, Lussier Y, Formenty P, Nichol MS, Feldmann H, Briese T, Lipkin WI. Panmicrobial oligonucleotide array for diagnosis of infectious diseases. Emerg Infect Dis. 2007;13(1):73-81.

42. Gardner SN, Jaing CJ, McLoughlin KS, Slezak TR. A microbial detection array (MDA) for viral and bacterial detection. BMC Genomics. 2010;11:668.

43. Cunningham SA, Mainella JM, Patel R. Misidentification of *Neisseria polysaccharea* as *Neisseria meningitidis* with the use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol. 2014;52(6):2270-1.

44. Desnos-Ollivier M, Blanc C, Garcia-Hermoso D, Hoinard D, Alanio A, Dromer F. Misidentification of *Saprochaete clavata* as *Magnusiomyces capitatus* in clinical isolates: utility of internal transcribed spacer sequencing and matrix-assisted laser desorption ionization-time of flight mass spectrometry and importance of reliable databases. J Clin Microbiol. 2014;52(6):2196-8.

45. Guo L, Ye L, Zhao Q, Ma Y, Yang J, Luo Y. Comparative study of MALDI-TOF MS and VITEK 2 in bacteria identification. J Thorac Dis. 2014;6(5):534-8.

46. Ecker DJ, Sampath R, Massire C, Blyn LB, Hall TA, Eshoo MW, Hofstadler SA. Ibis T5000: a universal biosensor approach for microbiology. Nat Rev Microbiol.

2008;6(7):553-8.

47. Wolk DM, Kaleta EJ, Wysocki VH. PCR-electrospray ionization mass spectrometry: the potential to change infectious disease diagnostics in clinical and public health laboratories. J Mol Diagn. 2012;14(4):295-304.

48. Metzgar D, Baynes D, Myers CA, Kammerer P, Unabia M, Faix DJ, Blair PJ. Initial identification and characterization of an emerging zoonotic influenza virus prior to pandemic spread. J Clin Microbiol. 2010;48(11):4228-34.

49. Bacconi A, Richmond GS, Baroldi MA, Laffler TG, Blyn LB, Carolan HE, Frinder MR, Toleno DM, Metzgar D, Gutierrez JR, Massire C, Rounds M, Kennel NJ, Rothman RE, Peterson S, Carroll KC, Wakefield T, Ecker DJ, Sampath R. Improved sensitivity for molecular detection of bacterial and Candida infections in blood. J Clin Microbiol. 2014;52(9):3164-74.

50. Jeng K, Hardick J, Rothman R, Yang S, Won H, Peterson S, Hsieh YH, Masek BJ, Carroll KC, Gaydos CA. Reverse transcription-PCR-electrospray ionization mass spectrometry for rapid detection of biothreat and common respiratory pathogens. J Clin Microbiol. 2013;51(10):3300-7.

51. Sampath R, Mulholland N, Blyn LB, Massire C, Whitehouse CA, Waybright N, Harter C, Bogan J, Miranda MS, Smith D, Baldwin C, Wolcott M, Norwood D, Kreft R, Frinder M, Lovari R, Yasuda I, Matthews H, Toleno D, Housley R, Duncan D, Li F, Warren R, Eshoo MW, Hall TA, Hofstadler SA, Ecker DJ. Comprehensive biothreat cluster identification by PCR/electrospray-ionization mass spectrometry. PLoS One. 2012;7(6):e36528.

52. Tevell Åberg A, Björnstad K, Hedeland M. Mass spectrometric detection of protein-based toxins. Biosecur Bioterror. 2013;11 Suppl 1:S215-26.

53. Domon B, Aebersold R. Mass spectrometry and protein analysis. Science 2006; 312(5771):212-7.

54. Boyer AE, Moura H, Woolfitt AR, Kalb SR, McWilliams LG, Pavlopoulos A, Schmidt JG, Ashley DL, Barr JR. From the mouse to the mass spectrometer: detection and differentiation of the endoproteinase activities of botulinum neurotoxins A-G by mass spectrometry. Anal Chem. 2005;77(13):3916-24.

55. Kalb SR, Moura H, Boyer AE, McWilliams LG, Pirkle JL, Barr JR. The use of Endopep-MS for the detection of botulinum toxins A, B, E, and F in serum and stool samples. Anal Biochem 2006;351(1):84-92.

56. Boyer AE, Quinn CP, Woolfitt AR, Pirkle JL, McWilliams LG, Stamey KL, Bagarozzi DA, Hart JC Jr, Barr JR. Detection and quantification of anthrax lethal factor in serum by mass spectrometry. Anal Chem. 2007;79(22):8463-70.

57. Boyer AE, Gallegos-Candela M, Quinn CP, Woolfitt AR, Brumlow JO, Isbell K, Hoffmaster AR, Lins RC, Barr JR. High-sensitivity MALDI-TOF MS quantification of anthrax lethal toxin for diagnostics and evaluation of medical countermeasures. Anal Bioanal Chem. 2015;407(10):2847-58.

58. Becher F, Duriez E, Volland H, Tabet JC, Ezan E. Detection of functional ricin by immunoaffinity and liquid chromatography-tandem mass spectrometry. Anal Chem 2007;79(2):659-65.

59. Kalb SR, Barr JR. Mass spectrometric detection of ricin and its activity in food and clinical samples. Anal Chem 2009;81(6):2037-42.

60. Kalb SR, Goodnough MC, Malizio CJ, Pirkle JL, Barr JR. Detection of botulinum neurotoxin A in a spiked milk sample with subtype identification through toxin proteomics.

Anal Chem 2005;77(19):6140-6.

61. Fredriksson SA, Hulst AG, Artursson E, de Jong AL, Nilsson C, van Baar BLM. Forensic identification of neat ricin and of ricin from crude castor bean extracts by mass spectrometry. Anal Chem 2005;77(6):1545-55.

62. Oestin A, Bergstrom T, Fredriksson S-A, Nilsson C. Solvent-assisted trypsin digestion of ricin for forensic identification by LC-ESI MS/MS. Anal Chem 2007;79(16):6271-8.

63. Fierz W. Basic problems of serological laboratory diagnosis. Mol Biotechnol. 1999;13(2):89-111.

64. Ito T, Kuwahara S, Yokota T. Automatic and manual latex agglutination tests for measurement of cholera toxin and heat-labile enterotoxin of *Escherichia coli*. J Clin Microbiol. 1983;17(1):7-12.

65. Nguyen M, Fries K, Khoury R, Zheng L, Hu B, Hildreth SW, Parkhill R, Warren W. Automated imaging and analysis of the hemagglutination inhibition assay. J Lab Autom. 2015 Oct 13. pii: 2211068215610061.

66. Peruski AH, Peruski LF Jr. Immunological methods for detection and identification of infectious disease and biological warfare agents. Clin Diagn Lab Immunol. 2003;10(4):506-13.

67. Mendoza LG, McQuary P, Mongan A, Gangadharan R, Brignac S, Eggers M. High-throughput microarray-based enzyme-linked immunosorbent assay (ELISA). Biotechniques. 1999;27(4):778-88.

68. Prince HE, Yeh C, Lapé-Nixon M. Utility of IgM/IgG ratio and IgG avidity for distinguishing primary and secondary dengue virus infections using sera collected more than 30 days after disease onset. Clin Vaccine Immunol. 2011;18(11):1951-6.

69. Ching KH, Lin A, McGarvey JA, Stanker LH, Hnasko R. Rapid and selective detection of botulinum neurotoxin serotype-A and -B with a single immunochromatographic test strip. J Immunol Methods. 2012;380(1-2):23-9.

70. Yonekita T, Ohtsuki R, Hojo E, Morishita N, Matsumoto T, Aizawa T, Morimatsu F. Development of a novel multiplex lateral flow assay using an antimicrobial peptide for the detection of Shiga toxin-producing *Escherichia coli*. J Microbiol Methods. 2013;93(3):251-6.

71. Lee JH, Seo HS, Kwon JH, Kim HT, Kwon KC, Sim SJ, Cha YJ, Lee J. Multiplex diagnosis of viral infectious diseases (AIDS, hepatitis C, and hepatitis A) based on point of care lateral flow assay using engineered proteinticles. Biosens Bioelectron. 2015;69:213-25.

72. Biodetection Technologies for First Responders: 2015 Edition. http:/biodetectionresource.pnnl.gov, (also http://www.tetracore.com/ and http://www.inbios.com/)

73. Slotved HC, Sparding N, Tanassi JT, Steenhard NR, Heegaard NH. Evaluating 6 ricin field detection assays. Biosecur Bioterror. 2014;12(4):186-9.

74. Slotved HC, Tanassi JT, Sparding N, Lindqvist A, Steenhard NR, Heegaard NH. Botulinum toxin field assays evaluated using cosmetic botox preparations. Biosecur Bioterror. 2013;11(4):280-6.

75. Hodge DR, Prentice KW, Ramage JG, Prezioso S, Gauthier C, Swanson T, Hastings R, Basavanna U, Datta S, Sharma SK, Garber EA, Staab A, Pettit D, Drumgoole R, Swaney E, Estacio PL, Elder IA, Kovacs G, Morse BS, Kellogg RB, Stanker L, Morse SA, Pillai SP. Comprehensive laboratory evaluation of a highly specific lateral flow assay for the

presumptive identification of ricin in suspicious white powders and environmental samples. Biosecur Bioterror. 2013;11(4):237-50.

76. Ramage JG, Prentice KW, Morse SA, Carter AJ, Datta S, Drumgoole R, Gargis SR, Griffin-Thomas L, Hastings R, Masri HP, Reed MS, Sharma SK, Singh AK, Swaney E, Swanson T, Gauthier C, Toney D, Pohl J, Shakamuri P, Stuchlik O, Elder IA, Estacio PL, Garber EA, Hojvat S, Kellogg RB, Kovacs G, Stanker L, Weigel L, Hodge DR, Pillai SP. Comprehensive laboratory evaluation of a specific lateral flow assay for the presumptive identification of abrin in suspicious white powders and environmental samples. Biosecur Bioterror. 2014;12(1):49-62.

77. Centers for Disease Control and Prevention. Evaluation of 11 commercially available rapid influenza diagnostic tests--United States, 2011-2012. MMWR Morb Mortal Wkly Rep. 2012;61(43):873-6.

78. Prendergast C, Papenburg J. Rapid antigen-based testing for respiratory syncytial virus: moving diagnostics from bench to bedside? Future Microbiol. 2013;8(4):435-44.

79. Fox JW, Cohen DM, Marcon MJ, Cotton WH, Bonsu BK. Performance of rapid streptococcal antigen testing varies by personnel. J Clin Microbiol. 2006;44(11):3918-22.

80. Rollins, G. The infectious diseases diagnostic revolution. Clinical Laboratory News, May 1, 2014.

81. Aggerbeck H, Nørgaard-Pedersen B, Heron I. Simultaneous quantitation of diphtheria and tetanus antibodies by double antigen, time-resolved fluorescence immunoassay. J Immunol Methods. 1996;190(2):171-83.

82. Yu H, Bruno JG. Immunomagnetic-electrochemiluminescent detection of *Escherichia coli* O157 and *Salmonella typhimurium* in foods and environmental water samples. Appl

Environ Microbiol. 1996;62(2):587-92.

83. Bruno JG, Yu H. Immunomagnetic-electrochemiluminescent detection of *Bacillus anthracis* spores in soil matrices. Appl Environ Microbiol. 1996;62(9):3474-6.

84. Wu D, Hugenholtz P, Mavromatis K, Pukall R, Dalin E, Ivanova NN, Kunin V, Goodwin L, Wu M, Tindall BJ, Hooper SD, Pati A, Lykidis A, Spring S, Anderson IJ, D'haeseleer P, Zemla A, Singer M, Lapidus A, Nolan M, Copeland A, Han C, Chen F, Cheng JF, Lucas S, Kerfeld C, Lang E, Gronow S, Chain P, Bruce D, Rubin EM, Kyrpides NC, Klenk HP, Eisen JA. A phylogeny-driven genomic encyclopaedia of Bacteria and Archaea. Nature. 2009;462(7276):1056-60.

85. Rinke C, Schwientek P, Sczyrba A, Ivanova NN, Anderson IJ, Cheng JF, Darling A, Malfatti S, Swan BK, Gies EA, Dodsworth JA, Hedlund BP, Tsiamis G, Sievert SM, Liu WT, Eisen JA, Hallam SJ, Kyrpides NC, Stepanauskas R, Rubin EM, Hugenholtz P, Woyke T. Insights into the phylogeny and coding potential of microbial dark matter. Nature. 2013;499(7459):431-7.

86. Fierer N, Breitbart M, Nulton J, Salamon P, Lozupone C, Jones R, Robeson M, Edwards RA, Felts B, Rayhawk S, Knight R, Rohwer F, Jackson RB. Metagenomic and small-subunit rRNA analyses reveal the genetic diversity of bacteria, archaea, fungi, and viruses in soil. Appl Environ Microbiol. 2007;73(21):7059-66.

87. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE. Metagenomic analysis of the human distal gut microbiome. Science. 2006;312(5778):1355-9.

88. NIH HMP Working Group, Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA, Bonazzi V, McEwen JE, Wetterstrand KA, Deal C, Baker CC, Di Francesco V,

Howcroft TK, Karp RW, Lunsford RD, Wellington CR, Belachew T, Wright M, Giblin C, David H, Mills M, Salomon R, Mullins C, Akolkar B, Begg L, Davis C, Grandison L, Humble M, Khalsa J, Little AR, Peavy H, Pontzer C, Portnoy M, Sayre MH, Starke-Reed P, Zakhari S, Read J, Watson B, Guyer M. The NIH Human Microbiome Project. Genome Res. 2009;19(12):2317-23.

89. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486(7402):207-14.

90. Hugon P, Dufour JC, Colson P, Fournier PE, Sallah K, Raoult D. A comprehensive repertoire of prokaryotic species identified in human beings. Lancet Infect Dis. 2015;15(10):1211-9.

91. Firth C, Lipkin WI. The genomics of emerging pathogens. Annu Rev Genomics Hum Genet. 2013;14:281-300.

92. Grard G, Fair JN, Lee D, Slikas E, Steffen I, Muyembe JJ, Sittler T, Veeraraghavan N, Ruby JG, Wang C, Makuwa M, Mulembakani P, Tesh RB, Mazet J, Rimoin AW, Taylor T, Schneider BS, Simmons G, Delwart E, Wolfe ND, Chiu CY, Leroy EM. A novel rhabdovirus associated with acute hemorrhagic fever in central Africa. PLoS Pathog. 2012;8(9):e1002924.

93. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N Engl J Med. 2012;367(19):1814-20.

94. Briese T, Paweska JT, McMullan LK, Hutchison SK, Street C, Palacios G, Khristova ML, Weyer J, Swanepoel R, Egholm M, Nichol ST, Lipkin WI. Genetic detection and characterization of Lujo virus, a new hemorrhagic fever-associated arenavirus from

southern Africa. PLoS Pathog. 2009;5(5):e1000455.

95. Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science. 2008;319(5866):1096-100.

96. Naccache SN, Peggs KS, Mattes FM, Phadke R, Garson JA, Grant P, Samayoa E, Federman S, Miller S, Lunn MP, Gant V, Chiu CY. Diagnosis of neuroinvasive astrovirus infection in an immunocompromised adult with encephalitis by unbiased next-generation sequencing. Clin Infect Dis. 2015;60(6):919-23.

97. Brown JR, Morfopoulou S, Hubb J, Emmett WA, Ip W, Shah D, Brooks T, Paine SM, Anderson G, Virasami A, Tong CY, Clark DA, Plagnol V, Jacques TS, Qasim W, Hubank M, Breuer J. Astrovirus VA1/HMO-C: an increasingly recognized neurotropic pathogen in immunocompromised patients. Clin Infect Dis. 2015;60(6):881-8.

98. Drosten C, Günther S, Preiser W, van der Werf S, Brodt HR, Becker S, Rabenau H, Panning M, Kolesnikova L, Fouchier RA, Berger A, Burguière AM, Cinatl J, Eickmann M, Escriou N, Grywna K, Kramme S, Manuguerra JC, Müller S, Rickerts V, Stürmer M, Vieth S, Klenk HD, Osterhaus AD, Schmitz H, Doerr HW. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med. 2003;348(20):1967-76.

99. Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, Tong S, Urbani C, Comer JA, Lim W, Rollin PE, Dowell SF, Ling AE, Humphrey CD, Shieh WJ, Guarner J, Paddock CD, Rota P, Fields B, DeRisi J, Yang JY, Cox N, Hughes JM, LeDuc JW, Bellini WJ, Anderson LJ; SARS Working Group. A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med. 2003;348(20):1953-66.

100. Svraka S, Rosario K, Duizer E, van der Avoort H, Breitbart M, Koopmans M. Metagenomic sequencing for virus identification in a public-health setting. J Gen Virol.

2010;91(11):2846-56.

101. Belák S, Karlsson OE, Blomström AL, Berg M, Granberg F. New viruses in veterinary medicine, detected by metagenomic approaches. Vet Microbiol. 2013;165(1-2):95-101.

102. Victoria JG, Kapoor A, Dupuis K, Schnurr DP, Delwart EL. Rapid identification of known and new RNA viruses from animal tissues. PLoS Pathog. 2008;4(9):e1000163.

103. Fredricks DN, Relman DA. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. Clin Microbiol Rev. 1996;9(1):18-33.

104. Brunstein J. Next generation sequencing and the search for emerging or unrecognized pathogens. MLO Med Lab Obs. 2014;46(12):26-7.

105. Hué S, Gray ER, Gall A, Katzourakis A, Tan CP, Houldcroft CJ, McLaren S, Pillay D, Futreal A, Garson JA, Pybus OG, Kellam P, Towers GJ. Disease-associated XMRV sequences are consistent with laboratory contamination. Retrovirology. 2010;7(1):111.

106. Naccache SN, Greninger AL, Lee D, Coffey LL, Phan T, Rein-Weston A, Aronsohn A, Hackett J Jr, Delwart EL, Chiu CY. The perils of pathogen discovery: origin of a novel parvovirus-like hybrid genome traced to nucleic acid extraction spin columns. J Virol. 2013;87(22):11966-77.

107. Strong MJ, Xu G, Morici L, Splinter Bon-Durant S, Baddoo M, Lin Z, Fewell C, Taylor CM, Flemington EK. Microbial contamination in next generation sequencing: implications for sequence-based analysis of clinical samples. PLoS Pathog. 2014;10(11):e1004437.

108. Kjartansdóttir KR, Friis-Nielsen J, Asplund M, Mollerup S, Mourier T, Jensen RH, Hansen TA, Rey-Iglesia A, Richter SR, Alquezar-Planas DE, Olsen PV, Vinner L, Fridholm H, Sicheritz-Pontén T, Nielsen LP, Brunak S, Willerslev E, Izarzugaza JM, Hansen AJ. Traces of ATCV-1 associated with laboratory component contamination. Proc Natl Acad Sci U S

A. 2015;112(9):E925-6.

109. Gruber K. Here, there, and everywhere: From PCRs to next-generation sequencing technologies and sequence databases, DNA contaminants creep in from the most unlikely places. EMBO Rep. 2015;16(8):898-901.

110. Allander T, Emerson SU, Engle RE, Purcell RH, Bukh J. A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species. Proc Natl Acad Sci U S A. 2001;98(20):11609-14.

111. Hall RJ, Wang J, Todd AK, Bissielo AB, Yen S, Strydom H, Moore NE, Ren X, Huang QS, Carter PE, Peacey M. Evaluation of rapid and simple techniques for the enrichment of viruses prior to metagenomic virus discovery. J Virol Methods. 2014;195:194-204.

112. Jensen RH, Mollerup S, Mourier T, Hansen TA, Fridholm H, Nielsen LP, Willerslev E, Hansen AJ, Vinner L. Target-dependent enrichment of virions determines the reduction of high-throughput sequencing in virus discovery. PLoS One. 2015;10(4):e0122636.

113. Oyola SO, Gu Y, Manske M, Otto TD, O'Brien J, Alcock D, Macinnis B, Berriman M, Newbold CI, Kwiatkowski DP, Swerdlow HP, Quail MA. Efficient depletion of host DNA contamination in malaria clinical sequencing. J Clin Microbiol. 2013;51(3):745-51.

114. Feehery GR, Yigit E, Oyola SO, Langhorst BW, Schmidt VT, Stewart FJ, Dimalanta ET, Amaral-Zettler LA, Davis T, Quail MA, Pradhan S. A method for selectively enriching microbial DNA from contaminating vertebrate host DNA. PLoS One. 2013;8(10):e76096.

115. Song Y, Giske CG, Gille-Johnson P, Emanuelsson O, Lundeberg J, Gyarmati P. Nuclease-assisted suppression of human DNA background in sepsis. PLoS One. 2014;9(7):e103610.

116. He S, Wurtzel O, Singh K, Froula JL, Yilmaz S, Tringe SG, Wang Z, Chen F, Lindquist EA, Sorek R, Hugenholtz P. Validation of two ribosomal RNA removal methods for

microbial metatranscriptomics. Nat Methods. 2010;7(10):807-12.

117. Mamanova L, Coffey AJ, Scott CE, Kozarewa I, Turner EH, Kumar A, Howard E, Shendure J, Turner DJ. Target-enrichment strategies for next-generation sequencing. Nat Methods. 2010;7(2):111-8.

118. Melnikov A, Galinsky K, Rogov P, Fennell T, Van Tyne D, Russ C, Daniels R, Barnes KG, Bochicchio J, Ndiaye D, Sene PD, Wirth DF, Nusbaum C, Volkman SK, Birren BW, Gnirke A, Neafsey DE. Hybrid selection for sequencing pathogen genomes from clinical samples. Genome Biol. 2011;12(8):R73.

119. Bright AT, Tewhey R, Abeles S, Chuquiyauri R, Llanos-Cuentas A, Ferreira MU, Schork NJ, Vinetz JM, Winzeler EA. Whole genome sequencing analysis of *Plasmodium vivax* using whole genome capture. BMC Genomics. 2012;13:262.

120. Carpi G, Walter KS, Bent SJ, Hoen AG, Diuk-Wasser M, Caccone A. Whole genome capture of vector-borne pathogens from mixed DNA samples: a case study of *Borrelia burgdorferi*. BMC Genomics. 2015;16:434.

121. Reyes GR, Kim JP. Sequence-independent, single-primer amplification (SISPA) of complex DNA populations. Mol Cell Probes. 1991;5(6):473-81.

122. Froussard P. A random-PCR method (rPCR) to construct whole cDNA library from low amounts of RNA. Nucleic Acids Res. 1992;20(11):2900.

123. Froussard P. rPCR: a powerful tool for random amplification of whole RNA sequences. PCR Methods Appl. 1993;2(3):185-90.

124. Grothues D, Cantor CR, Smith CL. PCR amplification of megabase DNA with tagged random primers (T-PCR). Nucleic Acids Res. 1993;21(5):1321-2.

125. Telenius H, Carter NP, Bebb CE, Nordenskjöld M, Ponder BA, Tunnacliffe A. Degenerate

oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. Genomics. 1992;13(3):718-25.

126. Zhang L, Cui X, Schmitt K, Hubert R, Navidi W, Arnheim N. Whole genome amplification from a single cell: implications for genetic analysis. Proc Natl Acad Sci U S A. 1992;89(13):5847-51.

127. Dean FB, Hosono S, Fang L, Wu X, Faruqi AF, Bray-Ward P, Sun Z, Zong Q, Du Y, Du J, Driscoll M, Song W, Kingsmore SF, Egholm M, Lasken RS. Comprehensive human genome amplification using multiple displacement amplification. Proc Natl Acad Sci U S A. 2002;99(8):5261-6.

128. Nelson JR, Cai YC, Giesler TL, Farchaus JW, Sundaram ST, Ortiz-Rivera M, Hosta LP, Hewitt PL, Mamone JA, Palaniappan C, Fuller CW. TempliPhi, phi29 DNA polymerase based rolling circle amplification of templates for DNA sequencing. Biotechniques. 2002;Suppl:44-7.

129. Langmore JP. Rubicon Genomics, Inc. Pharmacogenomics. 2002;3(4):557-60.

130. Barker DL, Hansen MS, Faruqi AF, Giannola D, Irsula OR, Lasken RS, Latterich M, Makarov V, Oliphant A, Pinter JH, Shen R, Sleptsova I, Ziehler W, Lai E. Two methods of whole-genome amplification enable accurate genotyping across a 2320-SNP linkage panel. Genome Res. 2004;14(5):901-7.

131. Park JW, Beaty TH, Boyce P, Scott AF, McIntosh I. Comparing whole-genome amplification methods and sources of biological samples for single-nucleotide polymorphism genotyping. Clin Chem. 2005;51(8):1520-3.

132. Bexfield N, Kellam P. Metagenomics and the molecular identification of novel viruses. Vet J. 2011;190(2):191-8.

133. Joensen KG, Tetzschner AM, Iguchi A, Aarestrup FM, Scheutz F. Rapid and Easy In Silico Serotyping of *Escherichia coli* Isolates by Use of Whole-Genome Sequencing Data. J Clin Microbiol. 2015;53(8):2410-26.

134. Huson DH, Auch AF, Qi J, Schuster SC. MEGAN analysis of metagenomic data. Genome Res. 2007;17(3):377-86.

135. Brady A, Salzberg SL: Phymm and PhymmBL: metagenomic phylogenetic classification with interpolated Markov models. Nat Methods. 2009;6(9):673–6.

136. Liu B, Gibbons T, Ghodsi M, Treangen T, Pop M. Accurate and fast estimation of taxonomic profiles from metagenomic shotgun sequences. BMC Genomics. 2011;12 Suppl 2:S4.

137. Segata N, Waldron L, Ballarini A, Narasimhan V, Jousson O, Huttenhower C. Metagenomic microbial community profiling using unique clade-specific marker genes. Nat Methods. 2012;9(8):811-4.

138. Rosen GL, Reichenberger ER, Rosenfeld AM. NBC: the Naive Bayes Classification tool webserver for taxonomic classification of metagenomic reads. Bioinformatics. 2011;27(1):127-9.

139. Patil KR, Haider P, Pope PB, Turnbaugh PJ, Morrison M, Scheffer T, McHardy AC. Taxonomic metagenome sequence assignment with structured output models. Nat Methods. 2011;8(3):191-2.

140. Ames SK, Hysom DA, Gardner SN, Lloyd GS, Gokhale MB, Allen JE. Scalable metagenomic taxonomy classification using a reference genome database. Bioinformatics. 2013;29(18):2253-60.

141. Wood DE, Salzberg SL: Kraken: ultrafast metagenomic sequence classification using exact

alignments. Genome Biol. 2014;15(3):R46.

142. Mande SS, Mohammed MH, Ghosh TS. Classification of metagenomic sequences: methods and challenges. Brief Bioinform. 2012;13(6):669-81.

143. Bazinet AL, Cummings MP. A comparative evaluation of sequence classification programs. BMC Bioinformatics. 2012;13:92.

144. Davenport CF, Neugebauer J, Beckmann N, Friedrich B, Kameri B, Kokott S, Paetow M, Siekmann B, Wieding-Drewes M, Wienhöfer M, Wolf S, Tümmler B, Ahlers V, Sprengel F. Genometa--a fast and accurate classifier for short metagenomic shotgun reads. PLoS One. 2012;7(8):e41224.

145. MacDonald NJ, Parks DH, Beiko RG. Rapid identification of high-confidence taxonomic assignments for metagenomic data. Nucleic Acids Res. 2012;40(14):e111.

146. Berendzen J, Bruno WJ, Cohn JD, Hengartner NW, Kuske CR, McMahon BH, Wolinsky MA, Xie G. Rapid phylogenetic and functional classification of short genomic fragments with signature peptides. BMC Res Notes. 2012;5:460.

147. Freitas TA, Li PE, Scholz MB, Chain PS. Accurate read-based metagenome characterization using a hierarchical suite of unique signatures. Nucleic Acids Res. 2015;43(10):e69.

148. Koslicki D, Foucart S, Rosen G. WGSQuikr: fast whole-genome shotgun metagenomic classification. PLoS One. 2014;9(3):e91784.

149. Angly FE, Willner D, Prieto-Davó A, Edwards RA, Schmieder R, Vega-Thurber R, Antonopoulos DA, Barott K, Cottrell MT, Desnues C, Dinsdale EA, Furlan M, Haynes M, Henn MR, Hu Y, Kirchman DL, McDole T, McPherson JD, Meyer F, Miller RM, Mundt E, Naviaux RK, Rodriguez-Mueller B, Stevens R, Wegley L, Zhang L, Zhu B, Rohwer F.

The GAAS metagenomic tool and its estimations of viral and microbial average genome size in four major biomes. PLoS Comput Biol. 2009;5(12):e1000593.

150. Beszteri B, Temperton B, Frickenhaus S, Giovannoni SJ. Average genome size: a potential source of bias in comparative metagenomics. ISME J. 2010;4(8):1075-7.
151. Xia LC, Cram JA, Chen T, Fuhrman JA, Sun F. Accurate genome relative abundance estimation based on shotgun metagenomic reads. PLoS One. 2011;6(12):e27992.
152. Frank JA, Sørensen SJ. Quantitative metagenomic analyses based on average genome size normalization. Appl Environ Microbiol. 2011;77(7):2513-21.
153. Ghosh TS, Mohammed MH, Komanduri D, Mande SS. ProViDE: A software tool for accurate estimation of viral diversity in metagenomic samples. Bioinformation. 2011;6(2):91-4.
154. Zhao G, Krishnamurthy S, Cai Z, Popov VL, Travassos da Rosa AP, Guzman H, Cao S, Virgin HW, Tesh RB, Wang D. Identification of novel viruses using VirusHunter--an automated data analysis pipeline. PLoS One. 2013;8(10):e78470.
155. Roux S, Tournayre J, Mahul A, Debroas D, Enault F. Metavir 2: new tools for viral metagenome comparison and assembled virome analysis. BMC Bioinformatics. 2014;15:76.
156. Skewes-Cox P, Sharpton TJ, Pollard KS, DeRisi JL. Profile hidden Markov models for the detection of viruses within metagenomic sequence data. PLoS One. 2014;9(8):e105067.
157. Daly GM, Leggett RM, Rowe W, Stubbs S, Wilkinson M, Ramirez-Gonzalez RH, Caccamo M, Bernal W, Heeney JL. Host Subtraction, Filtering and Assembly Validations for Novel Viral Discovery Using Next Generation Sequencing Data. PLoS One. 2015;10(6):e0129059.

158. Roux S, Enault F, Hurwitz BL, Sullivan MB. VirSorter: mining viral signal from microbial genomic data. PeerJ. 2015;3:e985.

159. Capobianchi MR, Giombini E, Rozera G. Next-generation sequencing technology in clinical virology. Clin Microbiol Infect. 2013;19(1):15-22.

160. Woo HJ, Reifman J. A quantitative quasispecies theory-based model of virus escape mutation under immune selection. Proc Natl Acad Sci U S A. 2012;109(32):12980-5.

161. Gire SK, Goba A, Andersen KG, Sealfon RS, Park DJ, Kanneh L, Jalloh S, Momoh M, Fullah M, Dudas G, Wohl S, Moses LM, Yozwiak NL, Winnicki S, Matranga CB, Malboeuf CM, Qu J, Gladden AD, Schaffner SF, Yang X, Jiang PP, Nekoui M, Colubri A, Coomber MR, Fonnie M, Moigboi A, Gbakie M, Kamara FK, Tucker V, Konuwa E, Saffa S, Sellu J, Jalloh AA, Kovoma A, Koninga J, Mustapha I, Kargbo K, Foday M, Yillah M, Kanneh F, Robert W, Massally JL, Chapman SB, Bochicchio J, Murphy C, Nusbaum C, Young S, Birren BW, Grant DS, Scheiffelin JS, Lander ES, Happi C, Gevao SM, Gnirke A, Rambaut A, Garry RF, Khan SH, Sabeti PC. Genomic surveillance elucidates Ebola virus origin and transmission during the 2014 outbreak. Science. 2014;345(6202):1369-72.

162. Park DJ, Dudas G, Wohl S, Goba A, Whitmer SL, Andersen KG, Sealfon RS, Ladner JT, Kugelman JR, Matranga CB, Winnicki SM, Qu J, Gire SK, Gladden-Young A, Jalloh S, Nosamiefan D, Yozwiak NL, Moses LM, Jiang PP, Lin AE, Schaffner SF, Bird B, Towner J, Mamoh M, Gbakie M, Kanneh L, Kargbo D, Massally JL, Kamara FK, Konuwa E, Sellu J, Jalloh AA, Mustapha I, Foday M, Yillah M, Erickson BR, Sealy T, Blau D, Paddock C, Brault A, Amman B, Basile J, Bearden S, Belser J, Bergeron E, Campbell S, Chakrabarti A, Dodd K, Flint M, Gibbons A, Goodman C, Klena J, McMullan L, Morgan L, Russell B, Salzer J, Sanchez A, Wang D, Jungreis I, Tomkins-Tinch C, Kislyuk A, Lin MF, Chapman

S, MacInnis B, Matthews A, Bochicchio J, Hensley LE, Kuhn JH, Nusbaum C, Schieffelin JS, Birren BW, Forget M, Nichol ST, Palacios GF, Ndiaye D, Happi C, Gevao SM, Vandi MA, Kargbo B, Holmes EC, Bedford T, Gnirke A, Ströher U, Rambaut A, Garry RF, Sabeti PC. Ebola Virus Epidemiology, Transmission, and Evolution during Seven Months in Sierra Leone. Cell. 2015;161(7):1516-26.

163. Tong YG, Shi WF, Liu D, Qian J, Liang L, Bo XC, Liu J, Ren HG, Fan H, Ni M, Sun Y, Jin Y, Teng Y, Li Z, Kargbo D, Dafae F, Kanu A, Chen CC, Lan ZH, Jiang H, Luo Y, Lu HJ, Zhang XG, Yang F, Hu Y, Cao YX, Deng YQ, Su HX, Sun Y, Liu WS, Wang Z, Wang CY, Bu ZY, Guo ZD, Zhang LB, Nie WM, Bai CQ, Sun CH, An XP, Xu PS, Zhang XL, Huang Y, Mi ZQ, Yu D, Yao HW, Feng Y, Xia ZP, Zheng XX, Yang ST, Lu B, Jiang JF, Kargbo B, He FC, Gao GF, Cao WC; China Mobile Laboratory Testing Team in Sierra Leone. Genetic diversity and evolutionary dynamics of Ebola virus in Sierra Leone. Nature. 2015;524(7563):93-6.

164. Forster M, Szymczak S, Ellinghaus D, Hemmrich G, Rühlemann M, Kraemer L, Mucha S, Wienbrandt L, Stanulla M; UFO Sequencing Consortium within I-BFM Study Group, Franke A. Vy-PER: eliminating false positive detection of virus integration events in next generation sequencing data. Sci Rep. 2015;5:11534.

165. Maldarelli F, Wu X, Su L, Simonetti FR, Shao W, Hill S, Spindler J, Ferris AL, Mellors JW, Kearney MF, Coffin JM, Hughes SH. HIV latency. Specific HIV integration sites are linked to clonal expansion and persistence of infected cells. Science. 2014;345(6193):179-83.

166. Ding D, Lou X, Hua D, Yu W, Li L, Wang J, Gao F, Zhao N, Ren G, Li L, Lin B. Recurrent targeted genes of hepatitis B virus in the liver cancer genomes identified by a

next-generation sequencing-based approach. PLoS Genet. 2012;8(12):e1003065.

167. Kwong JC, McCallum N, Sintchenko V, Howden BP. Whole genome sequencing in clinical and public health microbiology. Pathology. 2015;47(3):199-210.

168. Rusu LI, Wyres KL, Reumann M, Queiroz C, Bojovschi A, Conway T, Garg S, Edwards DJ, Hogg G, Holt KE. A platform for leveraging next generation sequencing for routine microbiology and public health use. Health Inf Sci Syst. 2015;3(Suppl 1 HISA Big Data in Biomedicine and Healthcare 2013 Con):S7.

169. Strong MJ, Xu G, Morici L, Splinter Bon-Durant S, Baddoo M, Lin Z, Fewell C, Taylor CM, Flemington EK. Microbial contamination in next generation sequencing: implications for sequence-based analysis of clinical samples. PLoS Pathog. 2014;10(11):e1004437.

170. Gargis AS, Kalman L, Berry MW, Bick DP, Dimmock DP, Hambuch T, Lu F, Lyon E, Voelkerding KV, Zehnbauer BA, Agarwala R, Bennett SF, Chen B, Chin EL, Compton JG, Das S, Farkas DH, Ferber MJ, Funke BH, Furtado MR, Ganova-Raeva LM, Geigenmüller U, Gunselman SJ, Hegde MR, Johnson PL, Kasarskis A, Kulkarni S, Lenk T, Liu CS, Manion M, Manolio TA, Mardis ER, Merker JD, Rajeevan MS, Reese MG, Rehm HL, Simen BB, Yeakley JM, Zook JM, Lubin IM. Assuring the quality of next-generation sequencing in clinical laboratory practice. Nat Biotechnol. 2012;30(11):1033-6.

171. Rehm HL, Bale SJ, Bayrak-Toydemir P, Berg JS, Brown KK, Deignan JL, Friez MJ, Funke BH, Hegde MR, Lyon E; Working Group of the American College of Medical Genetics and Genomics Laboratory Quality Assurance Commitee. ACMG clinical laboratory standards for next-generation sequencing. Genet Med. 2013;15(9):733-47.

172. Gargis AS, Kalman L, Bick DP, da Silva C, Dimmock DP, Funke BH, Gowrisankar S, Hegde MR, Kulkarni S, Mason CE, Nagarajan R, Voelkerding KV, Worthey EA, Aziz N,

Barnes J, Bennett SF, Bisht H, Church DM, Dimitrova Z, Gargis SR, Hafez N, Hambuch T, Hyland FC, Luna RA, MacCannell D, Mann T, McCluskey MR, McDaniel TK, Ganova-Raeva LM, Rehm HL, Reid J, Campo DS, Resnick RB, Ridge PG, Salit ML, Skums P, Wong LJ, Zehnbauer BA, Zook JM, Lubin IM. Good laboratory practice for clinical next-generation sequencing informatics pipelines. Nat Biotechnol. 2015;33(7):689-93.

173. Olson ND, Jackson SA, Lin NJ. Report from the Standards for Pathogen Identification via Next-Generation Sequencing (SPIN) Workshop. *Standards in Genomic Sciences*. 2015;10:119.

174. Fricke WF, Rasko DA. Bacterial genome sequencing in the clinic: bioinformatic challenges and solutions. Nat Rev Genet. 2014;15(1):49-55.

175. Köser CU, Ellington MJ, Cartwright EJ, Gillespie SH, Brown NM, Farrington M, Holden MT, Dougan G, Bentley SD, Parkhill J, Peacock SJ. Routine use of microbial whole genome sequencing in diagnostic and public health microbiology. PLoS Pathog. 2012;8(8):e1002824.

176. Didelot X, Bowden R, Wilson DJ, Peto TE, Crook DW. Transforming clinical microbiology with bacterial genome sequencing. Nat Rev Genet. 2012;13(9):601-12.

177. Köser CU, Bryant JM, Becq J, Török ME, Ellington MJ, Marti-Renom MA, Carmichael AJ, Parkhill J, Smith GP, Peacock SJ. Whole-genome sequencing for rapid susceptibility testing of M. *tuberculosis*. N Engl J Med. 2013;369(3):290-2.

178. Kostic AD, Ojesina AI, Pedamallu CS, Jung J, Verhaak RG, Getz G, Meyerson M. PathSeq: software to identify or discover microbes by deep sequencing of human tissue. Nat Biotechnol. 2011;29(5):393-6.

179. Borozan I, Wilson S, Blanchette P, Laflamme P, Watt SN, Krzyzanowski PM, Sircoulomb

F, Rottapel R, Branton PE, Ferretti V. CaPSID: a bioinformatics platform for computational pathogen sequence identification in human genomes and transcriptomes. BMC Bioinformatics. 2012;13:206.

180. Naeem R, Rashid M, Pain A. READSCAN: a fast and scalable pathogen discovery program with accurate genome relative abundance estimation. Bioinformatics. 2013;29(3):391-2.

181. Francis OE, Bendall M, Manimaran S, Hong C, Clement NL, Castro-Nallar E, Snell Q, Schaalje GB, Clement MJ, Crandall KA, Johnson WE. Pathoscope: species identification and strain attribution with unassembled sequencing data. Genome Res. 2013;23(10):1721-9

182. Xu G, Strong MJ, Lacey MR, Baribault C, Flemington EK, Taylor CM. RNA CoMPASS: a dual approach for pathogen and host transcriptome analysis of RNA-seq datasets. PLoS One. 2014;9(2):e89445.

183. Naccache SN, Federman S, Veeraraghavan N, Zaharia M, Lee D, Samayoa E, Bouquet J, Greninger AL, Luk KC, Enge B, Wadford DA, Messenger SL, Genrich GL, Pellegrino K, Grard G, Leroy E, Schneider BS, Fair JN, Martínez MA, Isa P, Crump JA, DeRisi JL, Sittler T, Hackett J Jr, Miller S, Chiu CY. A cloud-compatible bioinformatics pipeline for ultrarapid pathogen identification from next-generation sequencing of clinical samples. Genome Res. 2014;24(7):1180-92.

184. Byrd AL, Perez-Rogers JF, Manimaran S, Castro-Nallar E, Toma I, McCaffrey T, Siegel M, Benson G, Crandall KA, Johnson WE. Clinical PathoScope: rapid alignment and filtration for accurate pathogen identification in clinical samples using unassembled sequencing data. BMC Bioinformatics. 2014;15:262.

185. Rawat A, Engelthaler DM, Driebe EM, Keim P, Foster JT. MetaGeniE: characterizing

human clinical samples using deep metagenomic sequencing. PLoS One. 2014;9(11):e110915.

186. Rowe W, Baker KS, Verner-Jeffreys D, Baker-Austin C, Ryan JJ, Maskell D, Pearce G. Search engine for antimicrobial resistance: A cloud compatible pipeline and web interface for rapidly detecting antimicrobial resistance genes directly from sequence data. PLoS One. 2015;10(7):e0133492.
187. EDGE Bioinformatics. http://bsv.lanl.gov/edge.php
188. Langley G, Besser J, Iwamoto M, Lessa FC, Cronquist A, Skoff TH, Chaves S, Boxrud D, Pinner RW, Harrison LH. Effect of culture-independent diagnostic tests on future emerging infections program surveillance. Emerg Infect Dis. 2015;21(9):1582-8.
189. Bouricha M, Samad MA, Levy PY, Raoult D, Drancourt M. Point-of-care syndrome-based, rapid diagnosis of infections on commercial ships. J Travel Med. 2014;21(1):12-6.
190. Bradbury AR, Sidhu S, Dübel S, McCafferty J. Beyond natural antibodies: the power of in vitro display technologies. Nat Biotechnol. 2011;29(3):245-54.
191. Geyer CR, McCafferty J, Dübel S, Bradbury AR, Sidhu SS. Recombinant antibodies and in vitro selection technologies. Methods Mol Biol. 2012;901:11-32.
192. Bradbury A, Plückthun A. Reproducibility: Standardize antibodies used in research. Nature. 2015;518(7537):27-9.
193. Bradbury AM, Plückthun A. Antibodies: validate recombinants once. Nature. 2015;520(7547):295.
194. Lim DV, Simpson JM, Kearns EA, Kramer MF. Current and developing technologies for monitoring agents of bioterrorism and biowarfare. Clin Microbiol Rev. 2005;18(4):583-607.

Table 1. Summary of important features for NAATs

Criteria	Nucleic Acid Based (PCR and isothermal amplification)
Best Application Space	Environmental surveillance, clinical diagnostics, food and water safety. Limited utility for molecular epidemiology when detect (yes/no) is sufficient.
Other key advantages	Assay/throughput are highly scalable Does not require viability of pathogen Increased sensitivity over culture in some matrices (e.g. *Chlamydia* in urine)
Limitations	Poorly suited when PCR inhibitors are abundant and not adequately removed by sample prep. Can't readily distinguish live from dead pathogens. FDA-approved PCR assays for clinical diagnosis is limited for biothreat agents, but are available for research purposes.
Analytes Detected	DNA and RNA
Ability to Detect Unknowns	Only using universal primers, followed by sequencing or mass spectrometry.
Impact of Sample Matrices	Matrices with PCR inhibitors can be problematic if appropriate extraction protocol is not used. E.g. soils can contain varying amounts of humic acids which are known to inhibit PCR and blood can be problematic. Matrix specific specimen protocols are required for specific commercial tests.
Sensitivity	Analytical sensitivity is theoretically single copy, in practice 20-50 genome copies. Clinical sensitivity is different from analytical sensitivity, and in certain clinical applications it can be difficult to assess to the clinical sensitivity.
Specificity	Highly specific.
Reproducibility	Highly reproducible
Quantitative Ability	Highly quantitative, over 6 log for real-time PCR, which is excellent for relative quantitation. Absolute quantitation in real-time PCR requires a standard curve. Digital PCR permits very low quantitation below single copy levels per reaction. Isothermal methods are not reliably quantitative over the same range. Endpoint PCR methods such as Luminex and Genmark are also lacking in quantitative ability.
Ease of Use	Varies by method of nucleic acid amplification. Moderate complexity for qPCR, low complexity for most isothermal amplification based detection kits. The Alere™ i Influenza A & B, which is CLIA-Waived, is a good example of a low complexity test.
Lab based or Field deployable	Lab based for most qPCR, while some smaller battery powered units are field deployable. Many isothermal methods are both field deployable and POC.
Time to detection	Fast, less than 1 hr. Rate limiting step is extraction (sample prep).
Ease of interpretation	Yes/no results are less complex than quantitative results. All results must consider the possibility of contamination (or controls to exclude contamination). Clinical interpretation may also need consider detection of living vs. dead pathogen in cases following antibiotic therapy.
Throughput	From low (1-8 samples) for most isothermal based kits and field deployable battery operated instruments to high (96-384 or greater samples) for laboratory based instruments.

Cost per test	Varies per technology. As cheap as $1 per assay for bulk assays/reagents to $50 per test for some commercial assays. Reagent expiration, as for most methods can add cost.
Equipment investment	Approximately $50K for instrument without automated sample preparation. >$100K with automated sample prep. (Equipment investment is more than serology, but less than mass spec and NGS.)
Time and prerequisites to design new assay	Assay design requires knowledge of pathogen genomic sequences. Two weeks from sequence to deployed assays was possible for the SARS outbreak for public health emergency use.

Table 2. Summary of important features for Mass Spectrometry based Diagnostics

Criteria	Mass Spectrometry
Best Application Space	Clinical, epidemiology, forensics, food safety, universal and adaptable to many questions/problems
Other key advantages	MALDI - best for rapid screening, high throughput, and ease of use. e.g. Biotyper LC MS/MS; electrospray MS is best suited for untargeted (proteomics) and targeted, quantitation, sensitivity. Greater complexity than MALDI.
Limitations	Proper sample preparation for MS-based analysis is a critical step and can be both variable and time consuming. The quality and reproducibility of sample extraction and preparation significantly impact MS results, especially for non-targeted MS analysis.
Analytes Detected	Primarily proteins (e.g. toxins) and enzyme activity. But also chemical agents, formulations, viruses, bacteria and DNA mass analysis of PCR amplified amplicons (e.g. TIGR)
Ability to Detect Unknowns	Can detect both targeted knowns as well as untargeted unknowns.
Impact of Sample Matrices	Complex matrices not a problem for targeted detection of known. It is a limitation for untargeted MS analysis. But can also be an advantage for identifying extra formulation components.
Sensitivity	For targeted enzyme activity (attomoles/mL) For organism detection (Biotyper), 10e5 cells/µl For proteomics (femtomoles) Broad spectrum and targeted multiple reaction monitoring (MRM) (femtomoles)
Specificity	Highly specific. Specific identification of target and components, pathways, amino acid substitutions. Specific for actual protein being expressed. Specific identification of pathogen dependent on presence in database.
Reproducibility	Highly reproducible and precise. MS does not add random error into experiment.
Quantitative Ability	Yes for targeted isotope dilution and the quality of the quantitation is only dependent on the quality of the reference materials, quantitation for untargeted analytes is very complex and not a given.
Ease of Use	Full spectrum, targeted MALDI MS is quite easy. Untargeted MS are more complex and depends on the operator and the capabilities of the laboratory.
Lab based or Field deployable	Predominantly lab based, working towards portable versions. Current portable MS detectors are limited to specific chemical analytes and are not capable of analyzing unknowns or biologicals.
Time to detection	Sample prep is time limiting for targeted, time to first results 4-8 h for targeted, mass spec rate limiting 1-2 days for untargeted.
Ease of interpretation	Targeted (minutes), untargeted (1 day)
Throughput	Targeted (100-1000/day), untargeted (5/day)
Cost per test	Targeted $40, need more info from service providers, dependence on throughput, higher throughput reduces cost.
Equipment investment	$100K to $600K for low to high end instruments.

Time and prerequisites to design new assay	A month to deployment with validation for the best case, where standards are readily available.

Table 3. Summary of important features for Antibody/Antigen based Diagnostics

Criteria	Antibody/Antigen based assays
Best Application Space	Environmental, clinical, serological analysis, epidemiological analysis, food testing, water testing, forensics. Applicable wherever rapid, quick screening is needed. Poorly suited for unknown situation.
Other key advantages	Can provide detection of a specific host response. Can tune specificity based on selection of antibodies to be specific to a particular pathogen serotype or generic for a pathogen species or genus.
Limitations	Poorly suited for unknowns, unless you want to rule in or out specific agents.
Analytes Detected	Small molecules, proteins, lipids, carbohydrates, organisms, antibodies from immune response.
Ability to Detect Unknowns	Target specific assays – analytes restricted to the specificity of the antibody. Applicable to known antigens, organisms. Depends on the intended use, larger spectrum of antibodies can be designed.
Impact of Sample Matrices	Sample prep may be simpler than that required for other technologies. Therefore, impact of sample matrix maybe less. Used on any sample matrix. Preparation of sample is dependent on the target - intracellular v/s extracellular, membrane bound, whole microbe.
Sensitivity	Dependent on antibody used. Less sensitive than PCR or other nucleic acid based technologies. However, influenced by detection/readout format and assay chemistry. E.g. Suspension arrays v/s ELISA v/s lateral flow. Range of sensitivity is nM or pM. Varies from target to target. Depends on epitope selected.
Specificity	Dependent on quality of antibodies or antigens used. Specificity can be increased by sandwich assays employing separate capture and detection reagents. Combination of polyclonal and monoclonal used to balance sensitivity and specificity.
Reproducibility	Variable. Lot to lot antibody variation requires rigorous validation for each new lot. Likely to be resolved by a switch to recombinant sequenced antibodies.
Quantitative Ability	Usually standard curve run for reference. No internal standard. Depends on assay design. Semiquantitative. Is still an indirect measurement. Some disagreement on definition of "quantitative" assay. Is assay dependent. Sample loss or variability impacts quantitation. Depends on reference standard used. External reference standard accepted in analytical world.
Ease of Use	Depends on the assay chemistry and detection platform. Fairly standard protocols. LFA simple, ELISA maybe more complex.
Lab based or Field deployable	All applications possible.
Time to detection	Fairly rapid, 15-30 minutes for LFA, 2-4 hours for ELISA
Ease of interpretation	Fairly simple – visual interpretation for LFA, ELISAs use standard readers, have set thresholds.
Throughput	Low for LFA (1), medium to high – ELISAs, suspension arrays (100s to 1000s)
Cost per test	LFA $2-20 – one sample, no instrumentation ELISA - $10-$50 – depends on how many samples, analytes Suspension array - $30-$1000 – depends on how many samples, how many analytes.

Equipment investment	Instrument cost is 30-50K
Time and prerequisites to design new assay	Moderate to Complex. 3-6 months. Antibody development is the longest process, particularly because of the need to ensure specific binding.

Table 4. Summary of important features for Next Gen Sequencing based Diagnostics

Criteria	Next Gen Sequencing
Best Application Space	Epidemiology. Detection of unsuspected, emerging or unknown pathogens in clinical samples and vectors. Forensics. Poorly suited for environmental surveillance.
Other key advantages	Amplicon sequencing is ready for many applications; deep metagenomics is useful for basic research for detection of novel pathogens, including engineered organisms. The Illumina MiSeq and ION PGM platforms are useful for amplicon sequencing, while the HiSeq platform is appropriate for metagenomics. PacBio long-range sequencing is suitable for complete genome assembly.
Limitations	Poorly suited for environmental surveillance since genome coverage and sequence depth for pathogens will be very poor while the complexity of the normal diversity from natural environments will be quite high.
Analytes Detected	All DNA or RNA or both in any sample.
Ability to Detect Unknowns	Excellent ability to detect unknown samples
Impact of Sample Matrices	High impact of sample matrices on sample complexity as this increases the background genomic sequences
Sensitivity	Good.
Specificity	Need to target appropriate amplicons to achieve good specificity.
Reproducibility	Good, but dependent on bioinformatics analysis and standardized databases.
Quantitative Ability	Within a sample excellent; across samples can be difficult.
Ease of Use	Requires expertise
Lab based or Field deployable	Not currently field deployable, but perhaps in the future. Ability to analyze data remotely could be limiting depending on connectivity bandwidth
Time to detection	Day to week depending on sample prep and data analysis. Library prep can be rate limiting step.
Ease of interpretation	Varies – data from some samples are easy to interpret, while other samples require extensive analysis time. Can be limited by quality of available annotated sequence for a particular question, but this continues to improve over time.
Throughput	Scalable to an extent but library prep can be limiting for metagenomics uses.
Cost per test	Amplicon sequencing can be cost effective; metagenomics is costly. Cost benefit is realized on a higher scale.
Equipment investment	125K for MiSeq (amplicon sequencing), 750K for HiSeq (metagenomic).
Time and prerequisites to design new assay	Amplicon sequencing test can be designed in a few weeks (similar to PCR); metagenomics tests for all nucleic acid sequences.

Appendix 1. Workshop Participants

CDC and Public Health:	
Brett Austin	Ventura County Medical Center, CA
John Barr	Centers for Disease Control and Prevention
Ben Beard	Centers for Disease Control and Prevention
John Besser	Centers for Disease Control and Prevention
Thomas Blake	Centers for Disease Control and Prevention
Ann Boyer	Centers for Disease Control and Prevention
Jay Gee	Centers for Disease Control and Prevention
Alex Hoffmaster	Centers for Disease Control and Prevention
Suzanne Kalb	Centers for Disease Control and Prevention
Stephen Morse	Centers for Disease Control and Prevention
Ann Powers	Centers for Disease Control and Prevention
Linda Weigel	Centers for Disease Control and Prevention
Other HHS:	
Catharine Bosio	National Institute of Allergy and Infectious Diseases/NIH
Maureen Beanan	National Institute of Allergy and Infectious Diseases/NIH
Carol Linden	Biomedical Advanced Research and Development Authority/ASPR
DHS:	
Kevin Anderson	Department of Homeland Security
Dave Hodge	Department of Homeland Security
Segaran Pillai	Department of Homeland Security
National Laboratories:	
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Alina Deshpande	Los Alamos National Laboratory
Norman Doggett	Los Alamos National Laboratory
Kirsten McCabe	Los Alamos National Laboratory
Harshini Mukundan	Los Alamos National Laboratory
Dave Rakestraw	Lawrence Livermore National Laboratory
Tom Slezak	Lawrence Livermore National Laboratory
Karen Wahl	Pacific Northwest National Laboratory
Academia:	
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Jacque Fletcher	Oklahoma State University
Molly Hughes	University of Virginia
Paul Keim	Northern Arizona University
Elliot Lefkowitz	University of Alabama
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Patrick Martin	NVS Technologies, Inc.
Tom O'Brien	Tetracore, Inc.
Jamie Platt	Molecular Pathology Laboratory Network, Inc.
Kodumudi Venkat Venkateswaran	Omni Array Biotechnology, LLC.