ECRONICON OPEN ACCESS

EC MICROBIOLOGY
Review Article

The Nucleic Acids-Based Genetic Testing for Microorganisms: An Overview

Quanyi Li*

Development Services, BioReliance, USA

***Corresponding Author**: Quanyi Li, BioReliance, MilliporeSigma, Rockville, USA.

Received: March 16, 2017; **Published:** April 01, 2017

Abstract

Many genomes for important microorganisms including unculturable viruses and bacteria have been decoded and the sequences of the genomes are in the public databases. Additionally, many novel microorganisms are discovered using next generation sequencing technologies. With the known genomes and genes for the microorganisms, the nucleic acids-based genetic assays for detection and identification of the microorganisms have been developed. The nucleic acids-based genetic tests have been widely accepted for clinical diagnostics, food safety, environment and biosafety tests for biopharmaceutical products. Although the molecular tests can not differentiate live or dead microorganisms, the progress in viable PCR assays for microorganisms are very promising. The molecular testing has been used for the microorganism source tracking, monitoring and eventually elimination of microorganisms in a product or the environment. With the advanced knowledge of microorganism genomes, many developed molecular assays and the integration of automation in the biotech laboratories, it is most likely that the nucleic acids-based genetic testing for microorganisms will replace the culture-based testing for microorganisms in near future.

Keywords: *Molecular Assay; PCR; Sanger Sequencing; Next Generation Sequencing; Genetic Testing; Microorganisms*

Introduction

The culture-based tests for microorganisms are often referred as "golden standards" by microbiologists. The culture methods detect culturable microorganisms, but not unculturable microorganisms [1,2]. Microbiologists faced challenges in detecting slow growing bacteria such as *Mycobacterium* spp. and unculturable viruses such as norovirus for many years. Now, scientists detect those challenging human pathogens using molecular tests [3,4]. Over the past 30 years, microbiology has changed completely and become molecular microbiology era. When an unknown bacteria or virus is isolated and it can be sequenced immediately. With the sequence of the genome, a PCR assay can be developed for source tracking. One of examples was the outbreak of *Shiga*-toxin-producing *Escherichia coli* O104:H4 in 2011 in Germany [52]. The *E. coli* isolated was immediately sequenced with Ion Torrent next-generation sequencing (NGS), then the qPCR assay targeting the unique *Stx2a* allele was developed to detect the outbreak strain [53,54]. Rapidly, the contaminated food samples were found and discarded.

Recently, many new bacteria and viruses have been discovered using nucleic acids-based molecular methods and NGS. Many of the new microorganisms are accepted as new bacteria or virus species, even they remain as unculturable microorganisms. The small ssDNA virus family Parvoviridae has many new unculturable members including bovine parvovirus 2 and bovine parvovirus 3 [5,6]. The genomes of those bovine parvoviruses were detected in bovine serum products used in biopharmaceutical industries. Presence of the bovine viral nucleic acid sequences in bioproducts caused serious biosafety concern [7].

The sterility testing for microorganisms, *in vitro* cell culture assay with indicator cell lines for adventitious viruses are the standard GMP testing methods, and the molecular tests such as PCR and sequencing are used as additional supporting tests [8,9]. The nucleic acids-based genetic testing with PCR, Sanger DNA sequencing and next generation sequencing has significantly improved the testing quality such as higher sensitivity, better accuracy, and fast turn around time. With the use of advanced technologies and the quality compliance, the molecular tests can be the rapid test of choice for many laboratories [10,11].

1. Nucleic acids-based genetic testing technologies

The most popular molecular technologies used in genetic testing are PCR, DNA sequencing, and next generation sequencing (NGS). All those technologies are based on the principle of DNA replication. PCR amplifies trace amount of DNA or RNA molecules and generates million copies of amplicons. Since the invention of PCR in late 1980, PCR has become the most reliable and sensitive technology in detection of DNA or RNA molecules. Sanger DNA sequencing is used to identify a PCR product. Both PCR and Sanger DNA sequencing are used together for detection and identification of a microorganism. NGS provides entire genome analysis for one microorganism and metagenomics analysis for a community or population of microorganisms [55-57].

The molecular testing analyzes the targeted genes or genomes of the microorganisms with PCR, Sanger DNA sequencing and NGS. The molecular testing delivers rapid tests with high sensitivity and specificity. The molecular testing can be a presumptive or definite test [43,44]. When a molecular testing delivers the results with 100% certainty, the test is definite; and a presumptive test delivers the most likely results with significant confidence although the certainty is not 100%. Thus, molecular genetic testing is the most reliable test compared to culture-based tests.

1.1. PCR and applications in detection of microorganisms

There are many types of PCR including conventional PCR, real-time PCR, quantitative PCR, droplet digital PCR (ddPCR), and reverse transcription-PCR (RT-PCR) [12-15]. The conventional PCR is used for amplicon analysis such as amplicon fragment analysis, amplicon sequencing, nested PCR and multiple amplicons analysis. The conventional PCR is qualitative, or semi-quantitative. When using conventional PCR for detection of microorganisms, PCR product is sequenced and then used for BLAST search GenBank [58-60]. Recently, conventional PCR with degenerative primers was used to develop paramyxoviruses family PCR using capillary electrophoresis of amplicons for accurate sizing. The target gene was the conserved motif of viral polymerase [16]. Clearly, when conventional PCR delivers accurate sizing and sequencing of the targeted genes of microorganisms, it offers an excellent definite assay for detection and identification of broad range of microorganisms.

The real-time PCR is an amplification curve assay, and it uses the threshold of the number of PCR cycles (Ct). In real-time PCR, the amplification of DNA templates is monitored during the cycling of PCR reactions. The quantitation is achieved using the standard curves. The extreme specificity of DNA synthesis with Taq Polymerase makes the real-time PCR as the most popular quantitative assay. However, at the lower detection limit, the Ct value becomes unreliable and causes mis-interpretation [13]. Thus, single target real-time PCR detection of microorganisms is a presumptive assay. The positive test results should be verified by conventional PCR, DNA sequencing, or multiplex real-time PCR with additional targets [44,45]. The ddPCR performs in nanoliter droplets and the amplicons are counted and calculated at the end of PCR. The ddPCR delivers the standard curve independent absolute quantitation. The real-time PCR and ddPCR are the best assays for detection and quantitation of single or few targets of genes [12-15]. The RT-PCR targets the RNA molecule, and the RNA template is converted into cDNA with reverse transcriptase; then the cDNA templates are amplified by PCR. There are many viruses with RNA genomes and the RNA viruses can be detected by RT-PCR [37,45]. Furthermore, the RT-PCR can detect the expression of genes. Since RNA molecules have short life-time in bacteria, the RT-PCR was used to detect viable bacteria, not dead bacteria [80].

Standard PCR detects both viable and nonviable microorganisms. When the DNA from dead cells is damaged, the PCR may only detect DNA derived from viable microorganisms. Thus, viable PCR or vPCR was developed for detection of viable microorganisms. The viable PCR involves treatment of microorganisms in a sample with fluorescent dye which can penetrate damaged cells, but not intact viable cells. The dye then binds to cellular DNA and causes damage of DNA under light exposure. The damaged DNA subsequently cannot be amplified by PCR. By comparing the amplification curves (Ct values) between treated and untreated samples, the viable and nonviable microorganisms in the sample can be detected [17,18]. Alternatively, vPCR is used in detection of viable microorganism after enrichment culture. If a sample does not have viable microorganisms, the Ct values between cultured and uncultured samples would be similar. If a sample has viable microorganisms, the Ct value for cultured sample would be reduced due to the growth of viable microorganisms [19].

1.2. Sanger DNA sequencing and applications in identification of microorganisms

The Sanger DNA was developed by Fred Sanger in 1970. The Sanger sequencing offers the most reliable and accurate sequences [20]. Sanger sequencing can identify bacteria by sequencing 16S rRNA gene using bacteria universal primers and identify fungi by sequencing the ITS regions of rRNA genes using fungal universal primers [21-24]. The genetic identification of bacteria and fungi needs to have pure microorganism isolates for DNA extraction and then amplify 16S RNA gene or ITS region. The PCR products are purified and used for DNA sequencing with sequencing primers. Nowadays, sequencing of the 16S rRNA gene or ITS region is the first experiment for identification of unknown bacteria or fungi.

The DNA sequences can be analyzed using BLAST search against GenBank at National Center for Biotechnology Information (NCBI, www.ncbi.nim.nih.gov). The similarity of DNA sequences with GenBank entries are reported [61], therefore, the microorganisms are identified based on DNA sequences.

The genetic identification of bacteria and fungal isolates using DNA sequencing of 16S rRNA gene from bacteria and ITS regions form fungi has been commercialized as MicroSeq ID [25]. The MicroSeq ID is the standardized test which can be performed to GMP standards. Furthermore, other genes from microorganisms can be sequenced and used for typing or source tracking of the microorganisms. When a microorganism isolate is obtained, the strain can be determined using multilocus sequence typing (MLST) on PCR amplicons [26,27]. There are several international consortiums for tracking the sources of most important human pathogens using standardized MLST. One of examples, *Legionella pneumophila* sequence-based typing (SBT) is a standard test by EWIGLI (The European Working Group for *Legionella* Infections, www.ewigli.org). Total seven loci including *flaA, pilE, asd, mip, mpmpS, proA* and *neuA* are amplified by PCR and sequenced [28,29], and the variations of the sequences on the loci are called alleles. The allele profiles of the strain compared to the reference sequences are reported to EWIGLI where a database is maintained for international surveillance of the disease and monitoring the outbreak strains.

1.3. NGS and genome sequencing of microorganisms

The NGS can sequence millions of amplicons or small fragments in parallel using nanotechnologies [30,31]. The NGS can analyze a whole genome of microorganism or a population of microorganisms as metagenomics analysis [32,33]. With advanced bioinformatics tools and clouds computing, the NGS data can be processed rapidly with the return of vast number of reads. When a novel virus or adventitious virus was found by NGS universal virus screen [33,34], the presence of virus can be verified by PCR and Sanger sequencing of the amplicons so that the entirety of the assembling genome is confirmed. For identification of isolated virus or bacteriophage, the best approach is using NGS whole genome sequencing [34,35]. Bacteriophage contamination of *E. coli* fermentation for enzymes production is a serious manufacturing problem [91,92]. The bacteriophage genomes can be any type of nucleic acids such as ssDNA, dsDNA, ssRNA, and dsRNA [36]. The type of nucleic acids can be determined using enzyme digestion of the genomic nucleic acids. Then, a DNA or RNA library can be constructed with NGS adaptors and analyzed on either Ion Torrent or Illumina MiSeq. The reads can be assembled using virus or bacteriophage reference genomes.

NGS has been approved for genetic diagnosis by FDA [46,47]. NGS has become a powerful tool for personalized genomics in cancer diagnostics [46,47]. In many cases, Sanger sequencing was used for the confirmation test of genetic mutations detected by NGS [48,49]. The study of microorganisms using metagenomics with NGS has revealed many novel bacteria and viruses [31,50]. The research and early industrial entries for detection of adventitious viruses using NGS showed promising results [51]. Similarly, the novel bacteria and viruses detected by NGS must be confirmed for the entirety of the genomes using Sanger sequencing.

2. Development of molecular assays for detection and identification of microorganisms

There are many PCR assays developed for detection of microorganisms in clinical, food safety, environment and biosafety for biopharmaceutical products [62-65]. An assay development needs an assay concept, then the assay targets, and assay conditions. When an assay is developed, it needs to be validated and then it can be a test for the intended use.

2.1. The concept and targets for PCR assay

The concept comes from the intended use of the assay such as present or absent assay, quantitative assay; to detect a group of pathogens, a species, a strain, and a serogroup. When the intended use of the assay is decided, the identifier sequences are selected as the PCR target regions. The target regions must be unique for the bacteria group, species, or strain. When the PCR assays are for detection of strains, isolates, or serotypes; several target sequences are required. When the PCR targets are identified, the design of PCR primers and probes can be performed using bioinformatics tools. Then, PCR conditions are tested. The PCR positive standards either genomic DNA or RNA from the microorganism or synthesized target DNA sequences are used to evaluate the PCR primers, probes, and PCR conditions.

For qPCR assay for the toxigenic strain of *E. coli* O157: H7, there were several targets required, first target was to identify *E. coli* 157:H7 with *eae*, then with targets for *Shiga* toxin-1 (*stx1*) and *Shiga* toxin-2 (*stx2*); thus, a multiplex qPCR was used [66]. For qPCR assay for MRSA, two target components were used, one was for *Staphylococcus aureus* bacteria using the unique region Sa422, and another one was for the antibiotic resistant gene *mecA* [67]. Since *Legionella pneumophila* serogroup 1 causes most human infections, the qPCR assay for serogroup 1 has the clinical interest. The qPCR assay for serogroup 1 was developed using serogroup 1 strains specific targets from LPS cluster genes [68]. For fungi qPCR assays, the ITS sequences were selected to design primers and probes [24,69]. A qPCR assay for *Histoplasma capsulatum* was developed for clinical and environment testing; the human pathogen *Histoplasma capsulatum* cause lung infections through inhalation of fungal spores from air carrying fungal spores [70].

Viruses have diversified genome structures; each family or genus has the signature genome structures and genes. The conserved regions of viral polymerase genes were used to develop herpesvirus family PCR [71], and paramyxoviruses family PCR [16]. For enterovirus, 5' untranslated regions (5'UTR) were used to design the qPCR assay [72]. The viral capsids genes were used as PCR targets for RT-qPCR assays for genogroup I and II noroviruses [73].

2.2. Assay validation

When an assay is developed, the assay specifications need to be validated for the sensitivity, specificity, accuracy, precision, and reproducibility [39,74-76]. The suitability for type of samples or intended uses must be validated as well [74,75]. The validation can be performed by two analysts in single laboratory or in multiple laboratories. The purpose of validation is to prove the assay can be performed repeatedly.

For PCR assays, the primers must be verified electronically for the specificity using BLAST analysis or other bioinformatics tools. The PCR assays need to be performed in the presence of non-specific DNA, typically genomic DNA from bacteria, animal cells and plant tissues. The PCR assays do not amplify no-specific DNA. The sensitivities of the qPCR assay may be compared with another qPCR assay or with a culture method [77,78]. The accuracy needs to be evaluated with spiking of "known" amount of target DNA in various genomic DNA and matrix materials [74,75]. The linearity and slope of a qPCR standard curve are also indicators for accuracy [77,78]. The precision is another important criterion for a qPCR assay and it is expressed statistically using RSD (relative standard deviation) or coefficient of variation (CV) [74,75]. A good PCR assay must have less than 15% of CV [75]. Typically, qPCR has greater variation at lower limit of detection (LOD) and Limit of quantification (LOQ) [74,77]. When using the qPCR assay to test the target in a new matrix or product, a product qualification evaluation must be performed by spiking of known amounts of target [74,75] to prove that the spiked targets can be detected at low concentrations. In addition, an internal control for PCR assay, sample processing control with a microorganism or plasmid DNA to monitor the recovery are also required [74,75]. With a validated PCR assay, the performance of the assay is expected. For GMP testing, the assays must be validated [75].

2.3. Assay and test

A test works with its context, the intended use, the quality standards, the acceptance criteria, and the data interpretation. For example, EPA1615 is an official method developed by US EPA for drinking water test for Enteroviruses [37]. The test utilizes two type of assays, the qPCR assays for detection of viral genomes, and the culture method to access viable or non-viable virus using animal cell culture. The test

protocol covers the sampling procedure, RNA extraction protocol, qPCR assays and cell culture method, then data recording and analysis, result interpretation and reporting [37]. When molecular assays are used for testing of the microorganisms in clinical samples, food samples, environment samples and biopharmaceutical products, the intended use of the assays must be validated and approved by the regulatory agencies. Then, the molecular assays become regulated tests.

3. Quality systems and regulation requirements

The testing facility, lab and operation must compile with the quality standards known as ISO, cGLP and cGMP. The tests must meet the regulatory requirements including FDA and EPA. The testing laboratory must state clearly about the intended uses such as raw materials, bulk or product lot release tests [88]. The international standard document ISO17025 is one of the most important general requirements for the competence of testing and calibration laboratories. In ISO17025 document, the quality requirements have been clearly explained to cover the entire testing process [79].

In U.S., the microbiological testing including molecular testing is regulated and the corresponding quality standards are applied. The information about the regulatory organizations can be found on their websites [81-90]. In Europe, similar regulatory agencies and ISO standards are compiled for governing and guiding the testing industries. Microbiological tests for food safety are regulated by FDA, the official test methods are from FDA and AOAC validated methods [40]. When a test is developed, then it can be validated by AOAC, after FDA approval of the test, it becomes a FDA approved test method [40]. Similarly, GMP biosafety tests for adventitious microorganisms in biopharmaceutical products are FDA regulated tests, the tests must be validated and approved by FDA [41,42]. Clinical diagnostics are regulated by FDA and the corresponding regulatory agent CLIA [38,39].

Conclusion

The advances of genomics for all living microorganisms including both culturable and unculturable microorganisms have made the genetic testing for them a reality. Many of the unculturable microorganisms are known as molecular species and they can be potential risk and pathogens when they are present in environment, food, biopharmaceutical products. Our understanding about unculturable microorganisms is very limited. With the advanced molecular genetic testing for the microorganisms including both culturable and unculturable microorganisms, we can live in a safer environment with safer food, vaccines and biopharmaceutical products.

Conflict of Interest

The author declares no conflict of interest.

Bibliography

1. Wade W. "Unculturable bacteria-the uncharacterized organisms that cause oral infections". *Journal of the Royal Society of Medicine* 95.2 (2002): 81-83.

2. Mancini N., *et al*. "The era of molecular and other non-culture-based methods in diagnosis of sepsis". *Clinical Microbiology Review* 23.1 (2010): 235-251.

3. Peter-Gezlaff S., *et al*. "Detection and identification of Mycobacterium spp. in clinical specimens by combining the Roche Cobas Amplicor *Mycobacterium tuberculosis* assay with *Mycobacterium* genus detection and nucleic acid sequencing". *Journal of Clinical Microbiology* 48.11 (2010): 3943-3948.

4. Loisy F., *et al*. "Real-time RT-PCR for norovirus screening in shellfish". *Journal of Virological Methods* 123.1 (2005): 1-7.

5. Cotmore S., *et al*. "The family Parvoviridae". *Archives of Virology* 159.5 (2014): 1239-1247.

6. Allander T., *et al*. "A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species". *Proceedings of the National Academy of Sciences of the United States of America* 98.20 (2001): 11609-11614.

7. Marcus-Sekura C., *et al*. "Evaluation of the human host range of bovine and porcine viruses that may contaminate bovine serum and porcine trypsin used in the manufacture of biological products". *Biologicals* 39.6 (2011): 359-369.

8. US FDA. "Guidance for industry sterile drug products produced by aseptic processing - current Good Manufacturing Practice" (2004).

9. Gombolda J., *et al*. "Systematic evaluation of in vitro and in vivo adventitious virus assays for the detection of viral contamination of cell banks and biological products". *Vaccine* 32.24 (2014): 2916-2926.

10. Bloomfield MG. "Molecular testing for viral and bacterial enteric pathogens: gold standard for viruses, but don't let culture go just yet?" *Pathology* 47.3 (2015): 227-233.

11. Cronquist AB. "Impacts of culture-independent diagnostic practices on public health surveillance for bacterial enteric pathogens". *Clinical Infectious Diseases* 54.5 (2012): S432-S439.

12. Thermo Fisher Scientific online: Real-time PCR Handbook 4th Edition (2016).

13. Roche online: PCR Application Manual 3rd edition.

14. Sigma online: PCR Technologies Guide.

15. Bio-Rad online: Droplet Digital PCR Guide.

16. van Boheemen S., *et al*. "A family-wide RT-PCR Assay for detection of paramyxoviruses and application to a large-scale surveillance study". *PLOS One* 7.4 (2012): e34961.

17. Taskin B., *et al*. "Selective quantification of viable *Escherichia coli* bacteria in biosolids by quantitative PCR with propidium monoazide modification". *Applied and Environmental Microbiology* 77.13 (2011): 4329-4335.

18. Le'tant SE., *et al*. "Rapid-viability PCR method for detection of live, virulent *Bacillus anthracis* in environmental samples". *Applied and Environmental Microbiology* 77.18 (2011): 6570-6578.

19. Li B., *et al*. "Real-Time PCR methodology for selective detection of viable Escherichia coli O157:H7 cells by targeting Z3276 as a genetic marker". *Applied and Environmental Microbiology* 78.15 (2012): 5297-5304.

20. Thermo Fisher Scientific online: DNA sequencing by capillary electrophoresis Applied Biosystems chemistry guide, third edition.

21. Clarridge JE. "Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases". *Clinical Microbiology Review* 17.4 (2004): 840-862.

22. Janda JM., *et al*. "16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls". *Journal of Clinical Microbiology* 45.9 (2007): 2761-2764.

23. Fontana C., *et al*. "Use of the MicroSeq 500 16S rRNA gene-based sequencing for identification of bacterial isolates that commercial automated systems failed to identify correctly". *Journal of Clinical Microbiology* 43.2 (2005): 615-619.

24. Schocha CL., *et al*. "Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi". *Proceedings of the National Academy of Sciences of the United States of America* 109.6 (2012): 6241-6246.

25. Pryce TM., *et al*. "Rapid identification of fungi by sequencing the ITS l and ITS2 regions using an automated capillary electrophoresis system". *Medical Mycology* 41.5 (2003): 369-381.

26. Maiden MCJ., *et al*. "Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms". *Proceedings of the National Academy of Sciences of the United States of America* 95.6 (1998): 3140-3145.

27. Aanensen DM., *et al*. "The multilocus sequence typing network: mlst.net". *Nucleic Acids Research* 33.2 (2005): W728-W733.

28. Gaia V., *et al*. "Sequence-based typing of *Legionella pneumophila* serogroup 1 offers the potential for true portability in Legionellosis outbreak investigation". *Journal of Clinical Microbiology* 41.7 (2003): 2932-2939.

29. Ratzow S., *et al*. "Addition of neuA, the gene encoding N-acylneuraminate cytidylyl transferase, increases the discriminatory ability of the consensus sequence-based scheme for typing *Legionella pneumophila* serogroup 1 strains". *Journal of Clinical Microbiology* 45.6 (2007): 1965-1968.

30. Illumina online: An introduction to next generation sequencing technologies.

31. Whiteley AS., *et al*. "Microbial 16S rRNA Ion Tag and community metagenome sequencing using the Ion Torrent PGM Platform". *Journal of Microbiological Methods* 91.1 (2012): 80-88.

32. Quail MA., *et al*. "A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers". *BMC Genomics* 13 (2012): 341.

33. Ho T., *et al*. "Development of a virus detection and discovery pipeline using next generation sequencing". *Virology* 471-473 (2014): 54-60.

34. Daly GM., *et al*. "Host subtraction, filtering and assembly validations for novel viral discovery using next generation sequencing data". *PLOS One* 10.6 (2015): e0129059.

35. Zago M., *et al*. "Characterization of the genome of the dairy Lactobacillus helveticus bacteriophage ΦAQ113". *Applied and Environmental Microbiology* 79.15 (2013): 4712-4718.

36. Clokie MR., *et al*. "Bacteriophages: methods and protocols, Volume 1: isolation, characterization, and interactions". *Humana Press* (2010).

37. Fout GS., *et al*. "EPA Method 1615. Measurement of enterovirus and norovirus occurrence in water by culture and RT-qPCR. I. collection of virus samples". *Journal of Visualized Experiments* 97 (2015): 52067.

38. Liotta LA., *et al*. "Regulatory approval pathways for molecular diagnostic technology". *Methods in Molecular Biology* 823 (2012): 409-420.

39. Burd EM., *et al*. "Validation of laboratory-developed molecular assays for infectious diseases". *Clinical Microbiology Review* 23.3 (2010): 550-576.

40. Ceuppens S., *et al*. "Molecular methods in food safety microbiology: interpretation and implications of nucleic acid detection". *Comprehensive Reviews in Food Science and Food Safety* 13.4 (2014): 551-577.

41. Wisher M. "Biosafety and product release testing issues relevant to replication-competent oncolytic viruses". *Cancer Gene Therapy* 9.12 (2002): 1056-1061.

42. US FDA. "Guidance for industry-characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for Infectious disease indications" (2010).

43. CDC Online: Guidance for U.S. laboratories testing for Zika virus infection.

44. Fleming RI., *et al*. "The development of a mRNA multiplex RT-PCR assay for the definitive identification of body fluids". *Forensic Science International: Genetics* 4.4 (2010): 244-256.

45. Risatti G., *et al*. "Diagnostic evaluation of a real-time reverse transcriptase PCR assay for detection of classical swine fever virus". *Journal of Clinical Microbiology* 43.1 (2005): 468-471.

46. Evans BJ., *et al*. "The FDA and genomic tests — getting regulation right". *New England Journal of Medicine* 372.23 (2015): 2258-2264.

47. US FDA: "FDA advances Precision Medicine Initiative by issuing draft guidances on next generation sequencing-based tests" (2016).

48. Strom SP., *et al*. "Assessing the necessity of confirmatory testing for exome-sequencing results in a clinical molecular diagnostic laboratory". *Genetics in Medicine* 16.7 (2014): 510-515.

49. Mu W., *et al*. "Sanger confirmation is required to achieve optimal sensitivity and specificity in next-generation sequencing panel testing". *The Journal of Molecular Diagnostics* 18.6 (2016): 923-932.

50. Kohl C., *et al*. "Protocol for metagenomic virus detection in clinical specimens". *Emerging Infectious Diseases* 21.1 (2015): 48-57.

51. Mee ET., *et al*. "Development of a candidate reference material for adventitious virus detection in vaccine and biologicals manufacturing by deep sequencing". *Vaccine* 34.17 (2016): 2035-2043.

52. Mellmann A., *et al*. "Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology". *PLoS One* 6.7 (2011): e22751.

53. Rasko D., *et al*. "Origins of the *E. coli* strain causing an outbreak of hemolytic-uremic syndrome in Germany". *The New England Journal of Medicine* 365.8 (2011): 709-717.

54. Grad YH., *et al*. "Genomic epidemiology of the *Escherichia coli* O104:H4 outbreaks in Europe, 2011". *Proceedings of the National Academy of Sciences of the United States of America* 109.8 (2012): 3065-3070.

55. Kwong JC. *et al*. "Whole genome sequencing in clinical and public health microbiology". *Pathology* 47.3 (2015): 199-210.

56. Grumaz S., *et al*. "Next-generation sequencing diagnostics of bacteremia in septic patients". *Genome Medicine* 8.1 (2016): 73.

57. Mayo B., *et al*. "Impact of next generation sequencing techniques in food microbiology". *Current Genomics* 15.4 (2014): 293-309.

58. Frickmann H., *et al*. "16S rRNA gene sequence-based identification of bacteria in automatically incubated blood culture materials from tropical sub-Saharan Africa". *PLoS One* 10.8 (2015): e0135923.

59. Chen L., *et al*. "Rapid Sanger sequencing of the 16S rRNA gene for identification of some common pathogens". *PLoS One* 9.2 (2014): e88886.

60. Hu Z., *et al*. "Anchored pan dengue RT-PCR and fast Sanger sequencing for detection of Dengue RNA in human serum". *Journal of Medical Virology* 82 (2010): 1701-1710.

61. Altschul SF., *et al*. "Basic local alignment search tool". *Journal of Molecular Biology* 215.3 (1990): 403-410.

62. Espy EJ., *et al*. "Real-time PCR in clinical microbiology: applications for routine laboratory testing". *Clinical Microbiology Reviews* 19.1 (2006): 165-256.

63. Postollec F., *et al*. "Recent advances in quantitative PCR (qPCR) applications in food microbiology". *Food Microbiology* 28.5 (2011): 848-861.

64. Bej AK., *et al*. "Applications of the polymerase chain reaction in environmental microbiology". *PCR Methods and Applications* 1.3 (1992): 151-159.

65. Lovatt A. "Applications of quantitative PCR in the biosafety and genetic stability assessment of biotechnology products". *Reviews in Molecular Biotechnology* 82.3 (2002): 279-300.

66. Ibekwe AM., *et al*. "Detection and quantification of *Escherichia coli* O157:H7 in environmental samples by real-time PCR". *Journal of Applied Microbiology* 94.3 (2003): 421-431.

67. Reischl U., *et al*. "Rapid identification of methicillin-resistant *Staphylococcus aureus* and simultaneous species confirmation using real-time fluorescence PCR". *Journal of Clinical Microbiology* 38.6 (2000): 2429-2433.

68. Merault N., *et al*. "Specific real-time PCR for simultaneous detection and identification of *Legionella pneumophila* serogroup 1 in water and clinical samples". *Applied and Environmental Microbiology* 77.5 (2011): 1708-1717.

69. Toju H., *et al*. "High-coverage ITS primers for the DNA-based identification of Ascomycetes and Basidiomycetes in environmental samples". *PLoS ONE* 7.7 (2012): e40863.

70. De Leon MGF., *et al*. "Development of specific sequence-characterized amplified region markers for detecting *Histoplasma capsulatum* in clinical and environmental samples". *Journal of Clinical Microbiology* 50.3 (2012): 673-679.

71. Van Devanter DR., *et al*. "Detection and analysis of diverse herpesviral species by consensus primer PCR". *Journal of Clinical Microbiology* 34.7 (1996): 1666-1671.

72. Bennett S., *et al*. "Rapid simultaneous detection of enterovirus and parechovirus RNAs in clinical samples by one-step real-time reverse transcription-PCR assay". *Journal of Clinical Microbiology* 49.7 (2011): 2620-2624.

73. Vinje J., *et al*. "Development and application of a capsid VP1 (region D) based reverse transcription PCR assay for genotyping of genogroup I and II noroviruses". *Journal of Virological Methods* 116.2 (2004): 109-117.

74. EPA: Method validation of U.S. Environmental Protection Agency microbiological methods of analysis. FEM Document Number 2009-001 (2009).

75. FDA: Draft guidance for industry bioanalytical method validation (2013).

76. Raymaekers M., *et al*. "Checklist for optimization and validation of real-time PCR assays". *Journal of Clinical Laboratory Analysis* 23.3 (2009): 145-151.

77. Werling NJ., *et al*. "Systematic comparison and validation of quantitative real-time PCR methods for the quantitation of adeno-associated viral products". *Human Gene Therapy Methods* 26.3 (2015): 82-92.

78. Moens B., *et al*. "Development and validation of a multiplex real-time PCR assay for simultaneous genotyping and human T-lymphotropic virus type 1, 2, and 3 proviral load determination". *Journal of Clinical Microbiology* 47.11 (2009): 3682-3691.

79. ISO/IEC 17025: General requirements for the competence of testing and calibration laboratories. Reference number: ISO/IEC 17025:2005 (E).

80. Weigel KM., *et al*. "Molecular viability testing of bacterial pathogens from complex human sample matrix". *PLoS One* 8.1 (2013): e54886.

81. AACC: American Association of Clinical Chemistry for clinical diagnostics.

82. ANSI: American National Standards Institute for food safety and pharmaceutical products.

83. AOAC: AOAC International for food safety and pharmaceutical products.

84. ASTM: ASTM International for environment.

85. CLIA: Clinical Laboratory Improvement Amendments for clinical diagnostics.

86. CLSI: Clinical Laboratory Standards Institute for clinical diagnostics.

87. EPA: Environmental Protection Agency for environment.

88. FDA: Food and Drug Administration for food and pharmaceutical products.

89. ISO: International Organization for Standardization for food safety, environment and pharmaceutical products.

90. USP: United States Pharmacopeial Convention for pharmaceutical products.

91. Los M., *et al*. "Bacteriophage contamination: is there a simple method to reduce its deleterious effects in laboratory cultures and biotechnological factories?" *Journal of Applied Genetics* 45.1 (2004): 111-120.

92. Yaragalla S., *et al*. "PCR analysis: detection of bacteriophage contamination in biotechnology used to produce recombinant drugs". *Journal of Sciences* 3.1 (2012): 201-204.

Volume 7 Issue 3 April 2017