



Review Article

Molecular Microbiology in Clinical Practice: Current and Future Applications

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ABSTRACT

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Technological advances have yielded new molecular biology-based methods for the diagnosis of infectious diseases. The newest and most powerful molecular diagnostic tests are available at regional and national reference laboratories, as well as at specialized centers that are certified to conduct metagenomic testing. Metagenomic assays utilize advances in DNA extraction technology, DNA sequence library construction, high throughput DNA sequencing and automated data analysis to identify millions of individual strands of DNA extracted from clinical samples. At present, metagenomic assays are only possible at a small number of special research, academic and commercial laboratories. Continued research in human and pathogen genomic organization and host-pathogen interactions, represent important future goals that will maximize the information obtained from metagenomic assays. To illustrate the power and limitations of metagenomics, we report on a previously healthy 27 year old woman with work related exposure to ill animals, and who developed a rapidly progressive, severe diffuse interstitial pneumonitis that ultimately ended in the need for a double lung transplant. Metagenomic testing on DNA extracted from pleural fluid and nasopharyngeal swabs demonstrated the presence of expected normal bacterial flora along with some unexpected herpesvirus and non-HIV retroviral elements integrated into the patients DNA. Although no specific pathogen was ultimately identified to explain this patient's severe disease, the sample preparation and data analysis methods detailed herein illustrate the powerful benefits and limitations of metagenomic testing.

Introduction

During the past 25 years many extraordinary technological advances have yielded a multitude of molecular microbiology-based methods for the diagnosis and treatment of infectious diseases (1-6). Whereas once the diagnosis of patients with infections always required laborious special culture methods, waiting for pathogen growth in the lab, biochemical testing and in vitro determination of drug resistance, advances in molecular methods now can provide rapid results with highly reliable sensitivity and specificity. When first available, these tests were quite costly, however with time and

increased commercial availability from multiple vendors, the cost of such assays is becoming more affordable.

The decision by any institution to invest in an array of molecular diagnostics depends on many important factors: the number and frequency of assay requests, the cost of hardware and software and reagents specific to any one test, the ability of patients or institutions to pay for these tests, the importance of a rapid result, and the availability of so called "reference laboratories" that have higher capacities and thus a wider array of available tests with a reasonably short turnaround time. At our institution, the most commonly

utilized commercially available molecular microbiology diagnostic assays are summarized in Figure 1.

Diagnostic methods for patient care performed at clinical microbiology laboratories in the USA must be certified by CLIA, the Clinical Laboratory Improvement Amendment of the US Federal Centers for Medicare & Medicaid Services (CMS) (7). CLIA requires all labs that perform even one diagnostic test on human samples to meet certain Federal requirements. If an entity performs diagnostic tests, it is considered under CLIA to be a laboratory and must register with the CLIA program. International CLIA laboratory certificates also are available.

A second tier of “reference laboratories” exists for institutions that do not have a high enough volume of samples to make it economical to invest in the required lab infrastructure. Most hospitals have formal contractual relationships with a list of external reference laboratories. The wide range of reference laboratories includes local and state public health laboratories, larger referral center and private institutional clinical microbiology labs (e.g. Mayo Clinic, ARUP, Washington University, University of Washington Seattle, and the Centers for Disease Control, Labcorp Inc., Quest Diagnostics Inc, and the US National Institutes of Health in Bethesda, Maryland, USA. Specific assays are only approved to be performed on specific types of samples, and thus an assay approved for cerebrospinal fluid or a bronchoalveolar lavage, may not be appropriate, sensitive or specific enough to run on a blood or stool sample.

The future or “next generation” of powerful molecular diagnostic assays include various types of metagenomic assays – methods that utilize advances in DNA extraction technology, high throughput DNA sequencing and automated data analysis to identify millions of individual strands of DNA extracted from a clinical samples (8,9). These methods can differentiate viral, bacterial and/or parasitic DNA from background human DNA in complex clinical samples. The extraordinary power of these methods, not only in automated identification of organisms that are difficult or impossible to cultivate in the lab, also provide a glimpse into the human genome of the patient who is being studied. Metagenomic tests are presently done only at specialized centers of commercial laboratories, and the type of sample each lab is authorized to analyze differs greatly. Some labs are only authorized to process specific sample sources (e.g. blood) while others have CLIA certification to process more diverse sample sources such as urine, skin scraping, pleural or cerebrospinal fluid, etc.

Understanding the sensitivity and specificity of new assays is essential for clinicians who choose to use such technology. Sometimes, even with the application of the most advanced forms of molecular diagnostic tests, such as the metagenomic screening described in the case report detailed herein, results are inconclusive. However continued research into the basic science underlying host defense mechanisms, along with better understanding of human and pathogen genomic organization and host-pathogen interactions, represent important future goals that will maximize the value of metagenomic data.

The last three decades saw the emergence of many important but previously unrecognized viral illnesses - viruses such as Ebola, MERS (2012, Middle East Respiratory Syndrome) caused by a

Respiratory samples:

- Respiratory Extended Pathogen Panel Nucleic Acid Amplified test (NAAT) Adenovirus, Coronaviruses (SARS-CoV-2, 229E, HKU1, NL63, and OC43), Human Metapneumovirus (HMPV), Human Rhinovirus/Enterovirus, Influenza A, Influenza B, Parainfluenza 1, Parainfluenza 2, Parainfluenza 3, Parainfluenza 4, SARS-CoV-2 (2019-nCoV), and Respiratory Syncytial Virus (RSV)) and bacterial pathogens (Bordetella parapertussis, Bordetella pertussis, Chlamydia pneumoniae, and Mycoplasma pneumoniae).

- Atypical Pneumonia Panel Nucleic Acid Amplified Test (NAAT) Legionella pneumophila, Tatlockia (Legionella) micdadei, Mycoplasma pneumoniae, and Chlamydia pneumoniae

- Bordetella pertussis/parapertussis Nucleic Acid Amplified Test (NAAT) Bordetella pertussis/parapertussis

Cerebrospinal fluid

- Meningitis and Encephalitis Panel Nucleic Acid Amplification Test (NAAT): Escherichia coli K1, Haemophilus influenzae, Listeria monocytogenes, Neisseria meningitidis, Streptococcus agalactiae (Group B strep), Streptococcus pneumoniae, Cytomegalovirus (CMV), Enterovirus, Herpes Simplex Virus 1 (HSV-1), Herpes Simplex Virus 2 (HSV-2), Human Herpesvirus 6 (HHV-6), Human parechovirus, Varicella zoster virus (VZV), and Cryptococcus neoformans/gattii

Stool

- Clostridium Difficile Nucleic Acid Amplified Test with reflex to Toxin confirmation

- Clostridium difficile PCR positive will reflex to antigen testing

Sexually transmitted infections

- Herpes Simplex Virus Types 1 and 2 Nucleic Acid Amplified Test (NAAT) Herpes Simplex Virus 1 DNA, Herpes Simplex Virus 2 DNA

- Chlamydia trachomatis (CT)/Neisseria gonorrhoeae (NG) Nucleic Acid Amplification Test (NAAT) Chlamydia trachomatis DNA and Neisseria gonorrhoeae DNA

- HIV-1 RNA Quantitative Nucleic Acid Amplified Test (NAAT) HIV-1 RNA Copies/mL, HIV-1 RNA Log10 Copies/mL

- Trichomonas vaginalis Nucleic Acid Amplified Test (NAAT) Trichomonas vaginalis DNA

- Hepatitis C Quantitative Nucleic Acid Amplified Test (NAAT) Hepatitis C RNA IU/mL,

- Hepatitis C RNA Log10 IU/mL

Miscellaneous virus identifications

- BK Virus Nucleic Acid Amplified Test (NAAT) BK Plasma Log IU/mL and BK Virus Plasma Quantitative value in IU/mL

- Epstein Barr Virus Nucleic Acid Amplified Test (NAAT) Quantitative Epstein-Barr DNA Plasma Copies, Epstein-Bar DNA Plasma Log10 Copies, Epstein-Barr DNA Plasma IU, Epstein-Barr DNA Plasma Log IU

- Cytomegalovirus Nucleic Acid Amplified Test (NAAT) Qualitative

- Hepatitis B

Figure 1. Molecular microbiology tests performed in house at our institution, Froedtert and the Medical College of Wisconsin, Regional Medical Center, Milwaukee, Wisconsin, USA.

coronavirus, SARS (2002, Severe Acute Respiratory Syndrome caused by a different coronavirus), the current pandemic COVID19 (yet another coronavirus), Monkeypox and others (10-15). Especially in circumstances where standard methods do not identify a suspected pathogen and the number of possible pathogens is large, application of metagenomic testing is an extremely powerful tool. An example of the power and limitations of metagenomic testing is illustrated herein with a case report of a previously healthy woman who had extensive contact with sick animals and developed a rapidly progressive, nearly lethal pneumonitis and required a double lung transplant.

Case report

A 26 year-old previously healthy female was admitted to the hospital with shortness of breath, fevers and progressive interstitial pneumonitis. She was a student of veterinary medicine and worked with sick animals part time in small animal clinics in the midwestern United States (Wisconsin and Michigan) as well as on the island of Grenada in the Caribbean. She had not travelled outside of the USA for more than one year before she became ill, but had encountered a wide variety of sick small and large domestic animals. She was unaware of any coworkers who had a similar illness. Standard diagnostic assays for bacteria, fungi, parasites and viruses were all negative. Nonetheless there was concern that she had acquired an uncommon zoonotic viral infection that was beyond the capabilities of a standard US hospital based clinical microbiology laboratory. About one month prior to her illness, she was prescribed a short course of the antibiotic, trimethoprim-sulfamethoxazole (Bactrim™) to treat painful cysts, however she stopped that medication after just one day due to the development of a rash. She completed her cyst treatment using a fluoroquinolone antibiotic and recovered completely by the time of her hospital admission for respiratory failure.

Over the course of several weeks, her pulmonary function steadily deteriorated, she developed bilateral diffuse consolidative infiltrates and required intubation and mechanical ventilation with high oxygen concentrations with elevated positive end expiratory pressures to maintain adequate blood oxygenation. After several more weeks, mechanical ventilation failed to maintain adequate oxygenation so she was moved to the cardiac intensive care unit and administered ECMO (extra corporal membrane oxygenation), (16) via large bore catheters inserted into her venous and arterial systems. These efforts were complicated by both venous thromboembolisms despite anticoagulation and multiple pneumothoraxes. After two months on ECMO, she remained neurologically intact, she was fully alert and oriented and was able to participate in physical therapy and occupational therapy sessions. However her lungs did not recover and she developed densely consolidated lungs bilaterally consistent with rapidly progressive pulmonary fibrosis. The institutional organ transplant committee eventually concluded that her lungs had been irreparably damaged by an unknown cause so therefore she became a candidate for lung transplant. When a donor became available, she was transferred to another medical facility where she received a double lung transplant. At the time of this report, she was recovering well from surgery.

Metagenomic methods

Several nasal pharyngeal swabs and 5ml aliquots of pleural fluid obtained from her chest tubes were frozen at -800 C and used in a metagenomic testing algorithm that has proven valuable for identification of unknown viral, bacterial, fungal and parasitic zoonotic respiratory infections in the USA and globally (17-33). All standard microbiological testing available at our facility did not identify a pathogen. Fortunately, the high cost of metagenomic testing (presently \$5,000-\$8,000) of this patient's samples was minimized by working with a reference lab that was actively researching the etiology of pneumonitis in patients with animal exposures.

5 milliter aliquots of pleural fluid were centrifuged to process supernatants and cell pellets separately. Sufficient high quality nucleic acid (5-118 nanograms) was extracted from each pleural fluid sample and cell pellets, and nasopharyngeal swabs. DNA extracted from cell pellets, swabs and supernatants were analyzed separately. High quality sequencing libraries were generated from each DNA sample. DNA sequencing yielded the anticipated ~30 million reads per sample along with good quality DNA from positive control samples, and appropriately low background negative controls. The DNA extracted from nasal-pharyngeal swabs was sufficient to yield more than 30 million independent sequences for analysis. A subtraction method was utilized to enrich DNA samples for possible pathogen nucleic acids compared to human DNA. Data was analyzed for the presence of viruses, bacteria, parasites and fungi, as well as any viral elements that were incorporated into the patient's own DNA (20).

Results

Bacteria. No bacteria were detected in any of the pleural fluid samples. However the nasal swab contained a number of bacterial sequences representing normal oral flora: *Veillonella parvula*, *Actinomyces oris*, *Gemella haemolysans*, *Rothia* ssp., *Corynebacterium* ssp., *Streptococcus* ssp.

Fungi. A polymerase chain reaction (PCR) assay was used that targets the internal transcribed spacer (ITS) region of the fungal ribosomal RNA locus proven to identify basically all fungi. Primers were used for the ITS2 region for screening, which generates an amplification product of 375 nucleotides. No signal was obtained with any of the samples after 35 cycles of PCR. Similarly, no protozoan or metazoan parasite sequences were identified.

Viruses. A single sample revealed Epstein Barr Virus (human herpesvirus 4) DNA in the pleural fluid supernatants but not the corresponding cell pellets. On the other hand, several different endogenous retrovirus elements were identified in all samples (as expected for any human sample). Figure 2 shows the variety of viral elements found in this patient's samples.

Discussion

Metagenomic testing is an extraordinarily powerful method to explore the human "respiratory virome" when standard methods do not identify a known pathogen. Yet a major drawback at present is the expense and labor involved to yield reproducible data. However when applied judiciously, these methods have proven effective in identifying a wide array of viruses in the lungs of ill patients. The

Vietnam Initiative on Zoonotic Infections (VIZIONS) consortium of researchers has studied persons with severe respiratory distress after animal contacts in an effort to detect new or emerging infectious agents in humans and animals (23,24). The long-term goal of VIZIONS will assess how frequently new pathogens are circulating in human and animal populations and assess how frequently they are exchanged between species. In many of these studies, viruses previously not known to induce human disease have been identified. Whether or not all these new viruses will ever be considered human pathogens versus colonizers, remains to be determined. To date, this pioneering research has found numerous instances of expected human rhinoviruses, enteroviruses, influenza A virus, coronavirus OC43, and respiratory syncytial virus, rotavirus, Torque-teno virus, human papillomavirus, human beta herpesvirus 7, along with novel cyclovirus, vientovirus, gemycircularvirus, and statovirus (17-23). The blood DNA virome in several thousand asymptomatic humans has been reported by researchers in the USA and Singapore (Human Longevity Ltd.) 94 different viruses including DNA viruses, parvovirus and RNA viruses were identified in 42% of patients studied (34). RNA viruses included herpesviruses, anelloviruses, papillomaviruses, polyomaviruses, adenovirus, HIV, HTLV, hepatitis B and C, parvovirus B19 and influenza. Of relevance to transfusion medicine Merkel cell polyomavirus, papillomavirus and parvovirus were also identified. None of these aforementioned viruses were explained by contamination of commercial reagents or the environment (35,36). The potential for detection of rare environmental or contaminating DNA sequences emphasizes that the ultrasensitive metagenomic methods now available must be designed and interpreted with great caution.

Despite all our efforts, no single pathogenic microbe was identified in this patient prior to or after double lung transplant. For the purposes of the USA national lung transplant registry database, the diagnosis for this patient was entered as “rapidly progressive interstitial fibrosis, possibly secondary to an adverse immunological reaction to oral (Bactrim™, trimethoprim sulfamethoxazole)”. The same month as this patient’s hospitalization, the USA Food and Drug Administration added a new labelling warning for Bactrim™ users that in spite of its widely accepted use in tens of millions or persons across the USA for almost 50 years, in the past 10 years a small number of young patients on Bactrim™ were reported to develop progressive pulmonary dysfunction resulting in death, the need for ECMO or lung transplant (37). The absence of an infectious or clear autoimmune etiology for this patient’s illness was frustrating for all providers involved, however the results of metagenomic testing did reveal some curious information.

It is intriguing to speculate on the presence of latent herpesviruses such as EBV and endogenous retroviruses found in this patient. Although there is no clear pathogenic role of these organisms in patients with pulmonary fibrosis, there is considerable, sometimes contradictory clinical research ongoing that is exploring roles for latent human herpesvirus 8 (EBV) in the pathogenesis of pulmonary fibrosis (38,39). In our patient there was serological evidence of only two latent viruses - EBV early antigen IgG and CMV IgG, both common in most young healthy adults in the USA.

Lastly, the multiple strains of retroviruses found incorporated into this patient’s DNA, are well described in this era of human genome analysis. It has been well documented that the human genome includes repetitive DNA elements, among which human endogenous retroviruses (HERVs) account for about 8% (40). Evolutionary biologists consider HERVs to be DNA sequences of retroviral origin that have been acquired along the last 100 million of years through multiple integrations by now-extinct exogenous retroviruses. Furthermore, a direct immunostimulatory or immunosuppressive role for these elements have been reported in a number of studies. For example, an immunostimulatory role for HERV-W has been reported to play a role in dendritic and monocytic cell activation and the production of pro-inflammatory cytokines (41). In contrast, HERV-P71 and HERV-H have been reported to effect immune-suppression, including a Th1 to Th2 shift in immune tolerance and inhibition of T cell activation (42).

Conclusion

Although metagenomic testing of our patient’s pleural fluid and respiratory secretions did not identify a likely pathogen, this work nonetheless illustrates the extraordinary sensitivity and power of such next generation sequencing algorithms (43-45). We remain hopeful that once the high cost is lowered and the complex methods becomes more widely available, metagenomics will lead to many important new insights into the pathogenesis of new and emerging infectious diseases, as well as insights into immunopathological phenomena related to host-pathogen interactions.

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Conflicts of interest

The authors declare no conflicts of interest

Family	Genus	Species	IPS-567_NPS	IPS-568-PFL-F	IPS-568-PFL-S	IPS-568-PFR-I	IPS-568-PFR-I
Herpesviridae	Cytomegalovirus	Stealth virus 1	8	0	3	7	4
Herpesviridae	Lymphocryptovirus	Human gammaherpesvirus 4	339	0	5	0	2
Papillomaviridae	Betapapillomavirus	Human papillomavirus type 5b	3	0	2	5	7
Retroviridae	Lentivirus	Human immunodeficiency virus 1	35	4	58	40	47
Retroviridae	Retro_unclassified	Human endogenous retrovirus HCML-ARV	3	0	9	8	5
Retroviridae	Retro_unclassified	Human endogenous retrovirus HERV-K(II)	7	1	10	3	9
Retroviridae	Retro_unclassified	Human endogenous retrovirus KC4	8	1	16	8	7
Retroviridae	Retro_unclassified	Human endogenous retrovirus K	75	0	134	117	123
Retroviridae	Retro_unclassified	Human endogenous retrovirus	28	1	54	35	60
Retroviridae	Retro_unclassified	Multiple sclerosis associated retrovirus element	5	0	9	10	9

Figure 2. Output from metagenomic testing revealed a variety of Herpesviruses, Papilloma viruses and ancient non HIV/HTLV endogenous retroviral elements integrated into this patient’s DNA

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