CASE REPORTS

Applied Use of Next Generation DNA Sequencing for Infectious Disease

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Abstract:

The advent of high throughput human DNA sequencing capability has allowed a crossover for sequencing infectious diseases. The same technologies that allow us to query the human genome for cancer mutations, pharmacogenomics, and inherited genetic errors now allow a more in-depth analysis of human samples for evidence of infectious disease. Next Generation DNA sequencing (NGS) for infectious disease holds the promise of accuracy with greater sensitivity and specificity than culture, serologic and PCR methods. NGS allows for better discrimination between strains, species, detection of novel variants and new organisms, detection of an ever-growing array of uncultivable organisms, and the ability to detect eukaryotes that were previously undetectable. NGS also may soon provide the ability to determine drug resistance and sensitivity information. The following describes the Rapid Infectious Disease Identification System (RIDI™) and its practical use. Application of the RIDI™ system is discussed in four case reports with patients suffering from chronic malaise, rheumatoid arthritis, osteoarthritis, and chronic fatigue syndrome.

Keywords: next-generation sequencing for infectious disease, RIDI™, chronic fatigue syndrome, osteoarthritis, rheumatoid arthritis, *Funneliformis mosseae*, *Saccharomyces cerevisiae*, *Toxoplasma gondii*

Introduction:

Technology has historically been the harbinger of advances in medical science. Laboratory microbiology was initiated with the discovery of "animalcules" by Antony van Leuwenhoek, a Dutch scientist in the late 1600's. The further development of microscopy, culture methods, serologies, and more recently molecular techniques, have advanced our knowledge of microbes. Our improved understanding of microbes is an essential guide to pathogenesis, treatment, and cure of disease.

Molecular techniques, in many cases are becoming the primary technology used for infectious disease identification, especially for viral diseases such as HIV and Hepatitis A/B/C. Multiplex PCR systems, such as the Biofire system (BioMérieux), are in routine use with six panels including common respiratory infections and stool pathogens. In some hospital laboratories, multiplex PCR systems have supplanted both microscopy and culture of stool. While culture methods are important, especially in wound and sepsis identification, these technologies still have deficits, especially in sepsis where we are only able to culture 40-60% of bacteria causing sepsis (1-5). Sequencing and advanced molecular techniques may increase microbial

identification in sepsis to over 90%. Rapid detection is predicted to reduce hospitalization cost by an average of $20,000, a reduction of in-patient stay by 2 days and significantly reduce morbidity and mortality (1, 6, 7). Sequencing technology provides a clearer insight into the mix of flora in chronic wounds and allows an assessment of fungal contribution without the need for extended culture periods.

There are a variety of platforms used for sequencing. The Ion Torrent by Life Technologies/ThermoFisher is characterized by the Personal Genome Machine (PGM) and the Ion S5. The Illumina platforms include the MiSeq, HiSeq and soon the Firefly. Other sequencing platforms with practical applications in use or pending are the MinION by Oxford Nanopore, the Pac Bio RS II and Sequel by Pacific Biosciences, Gene Reader by Qiagen, and Genia by Roche. These systems dramatically enhance the capability of detection of a wider array of microorganisms when combined with an expanded microbial database. The need for this enlarged microbial database is illustrated by the tree of life (*Figure 1*) with an extensive variety and constellation of organisms that is rapidly expanding. Only now are these genetic fingerprints routinely detectable using NGS. The RIDI™ system developed by our laboratory is platform agnostic, with compatibility for use with any sequencer (Ion Torrent, MiSeq and PacBio). The cost per clinical sample has been a major factor, but this expense is steadily declining as the capacity of these systems develop with advancements in liquid handling, components, chemistry, automation and bioinformatics. With proper coding, infectious disease sequencing services are reimbursable by the Centers for Medicare and Medicaid Services (CMS) and most insurance companies.

Methods - New technologies and how they work:

The RIDI™ system relies on an amplicon strategy rather than an untargeted strategy for molecular annealing thus allowing increased sensitivity and specificity. The system utilizes patented prokaryotic (bacterial) and eukaryotic (fungal, protozoal, algal) barcoded DNA primers that 'glue' or anneal to the suspect microbe's DNA after the total DNA (microbial and host) has been extracted from the patient sample. The amplification is performed using conventional PCR. This system does not require microbial culture. The amplicons, (many trillions of copies of DNA), are pooled creating a library. ISP's (ion sphere particles) are then created from the pooled DNA library and are then loaded onto an Ion Torrent semiconductor chip. The chip detects DNA copying or extension in microwells using an ISFET (ion sensitive fixed effect transistor) that reads pH changes as each base pair is added. The signal from the chip is then processed by software that seamlessly integrates sample identification, sequencing, sequence analysis, comparison to a curated database, and then generates a final clinical report. The database relies on the NCBI (National Center for Biotechnology Information) database which includes 16s and 18s microbes with over 48,000,000 DNA sequence entries linked to a specific life form. The advantage of using a comprehensive database is that it supports a non-exclusionary identification approach and is continually updated. This allows an unbiased approach for organism detection and allows the RIDI™ system to have a high degree of sensitivity and specificity. The sensitivity and specificity of the RIDI™ system using an *E. coli* standard are both greater than 95% and is able to detect 146 cells/mL (9). A sequencing run using this system with the Ion Torrent sequencer can be completed in 12 hours from start to finish, while proof-of-concept from extraction to data analysis of a sequencing

run will soon allow single shift capacity in the clinical microbiology laboratory. The assay is continually challenged and validated by reference samples from the following institutions; API (American Proficiency Institute), CAP (College of American Pathology), ATCC (American Type Culture Collection), and BEI (Biodefense and Emerging Infections Research Resources Repository). Our laboratory is currently working with industry leaders to develop international reference standards for others conducting NGS for infectious disease.

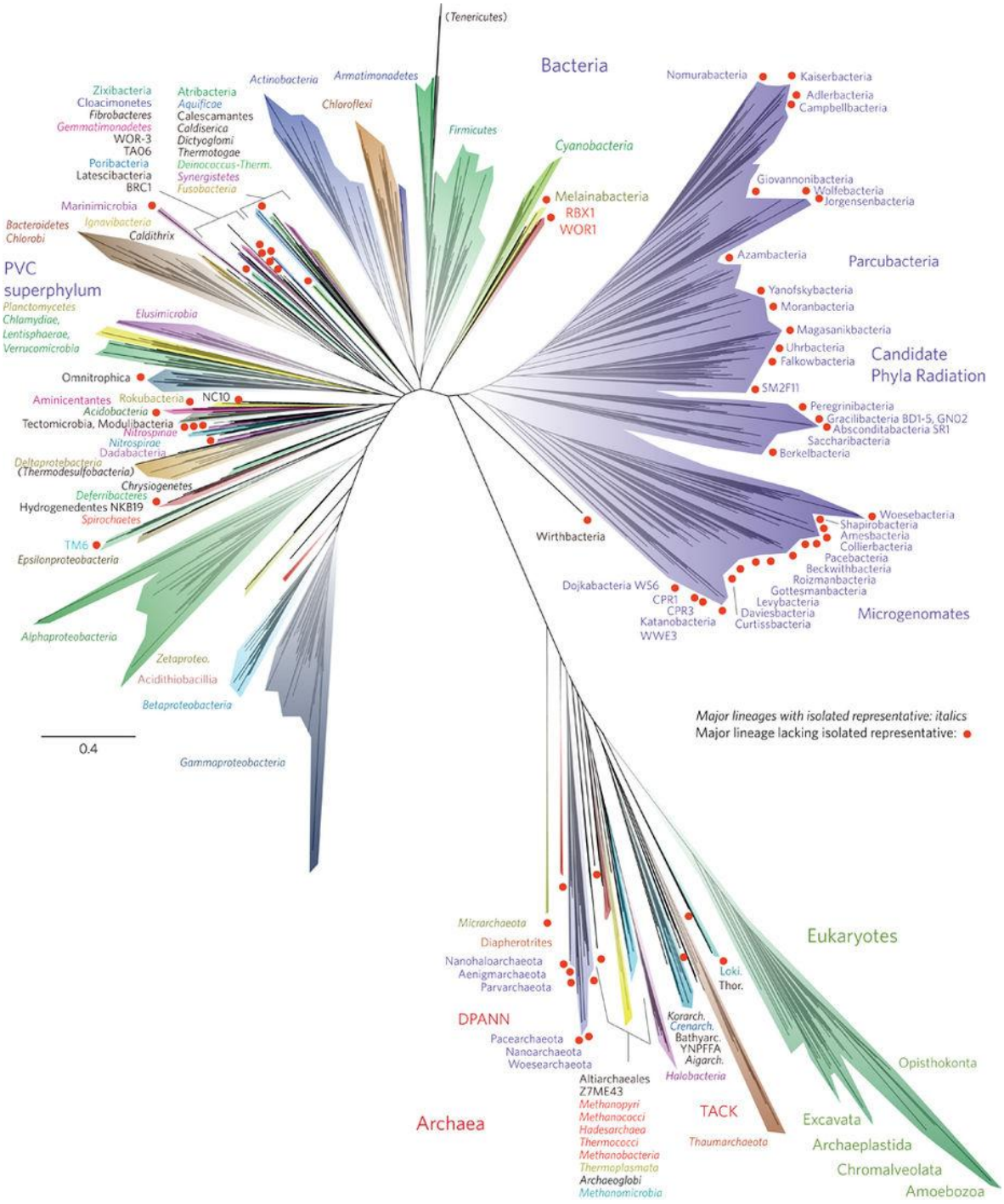


Figure 1. Phylogenetic Tree. Distribution of the diversity of life across the bacteria, archaea, and eukaryotic domains. (8).

The RIDI™ system is available clinically from our laboratory, but we are developing a kit that will allow the system to be distributed commercially on other sequencing platforms for clinical laboratories worldwide. Other sequencing systems are available, but they cost more, use restricted and overly curated databases, require preceding culture steps, have longer run times, or require samples relatively free of host DNA (such as serum instead of whole blood). The RIDI™ system has been available for clinical use by Fry Laboratories, LLC for over 4 years. Acceptable sample types include whole blood, sputum, bone, cerebral spinal fluid, joint fluid, exudates, stool, and urine; all of which do not require preceding microbial culture.

In the case reports presented; histologic stains using Giemsa and a proprietary fluorescent DNA stain were utilized, photomicrographs were taken using a Nikon Eclipse E600 at 400-1000X. Fluorescent emission microscopy was performed using the same microscope at 461 nm. Case #1 was from a sample received for analysis in our diagnostic laboratory, cases 2-4 were seen in the author's clinical practice, all patients provided informed consent.

Case reports:

#1 'Chronically Ill'

Our laboratory received a peripheral blood sample from a chronically ill 30-year-old Caucasian female patient. The referring physician had ordered Giemsa stains for detection of possible vector-borne diseases. The sample was received, the blood smear stained with Giemsa displayed an abundance of rod shaped bacteria (*Figure 2*). The treating physician was notified, and blood cultures were ordered from a regional microbiology laboratory. Culture results determined the presence of *Enterobacter cloacae* complex 4 days later (10). The complex may be composed of the following microbes that are not easily distinguishable by standard microbiology methods:

- *Enterobacter cloacae*
- *Enterobacter hormaechei*
- *Enterobacter asburiae*
- *Enterobacter kobei*
- *Enterobacter ludwigii*
- *Enterobacter nimipressuralis.*

To aid in the accuracy of the diagnosis, bacterial DNA sequencing was conducted using the RIDI™ system. The system identified, more specifically, *Enterobacter ludwigii*. (*Figure 3*). The patient was started on broad-spectrum IV antibiotics and then switched to levofloxacin once identification was made.

This is an example of a more precise diagnosis by molecular techniques as well as demonstrating the speed to diagnosis by Next-Generation DNA Sequencing using the RIDI™ system. Comparatively, culture systems when positive may take 3-7 days for identification. *Enterobacter cloacae* complex consists of a variety of organisms, while NGS clarified the microbial population more distinctly and in an ideal situation could have superseded culture identification by days. Microscopy, culture, and NGS dramatically illustrate a non-septic bacteremia.

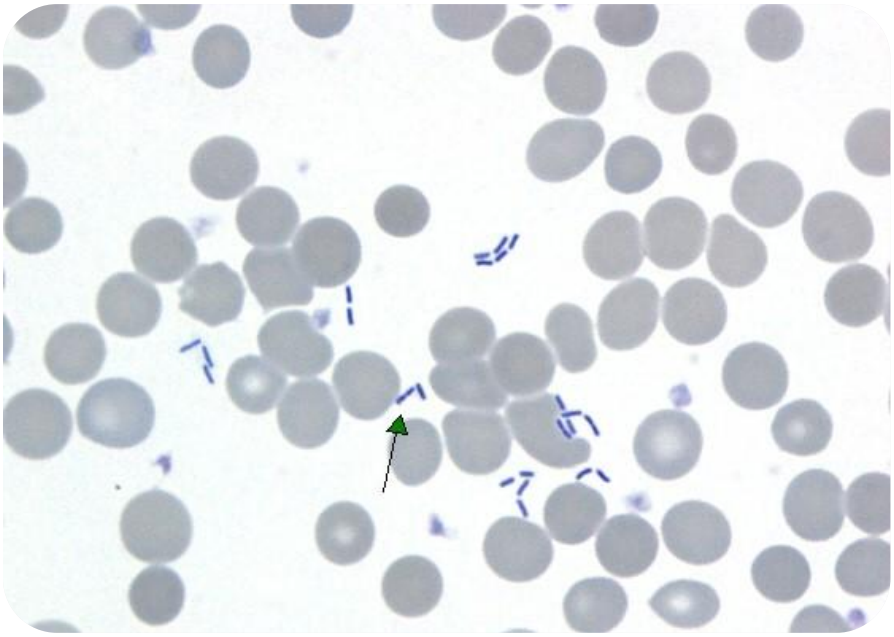


Figure 2. Peripheral blood smear photomicrograph. Giemsa stain of peripheral blood smear indicated a significant number of rod-shaped organisms, 1,000X .

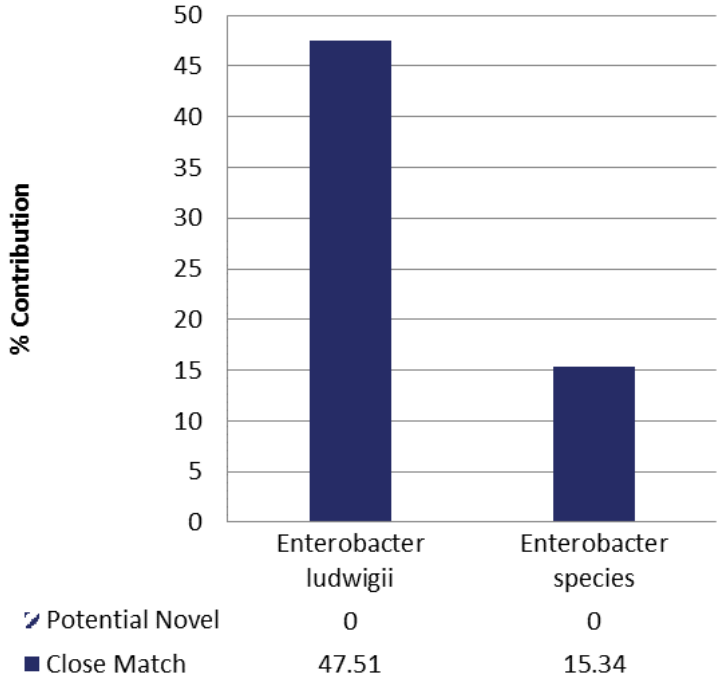


Figure 3. DNA Sequencing Results for bacteremia. *Enterobacter ludwigii* was detected via NGS, while bacterial culture reported the more general result of the *Enterobacter cloaceae* complex.

#2 Rapid Onset Rheumatoid Arthritis with Toxoplamosis

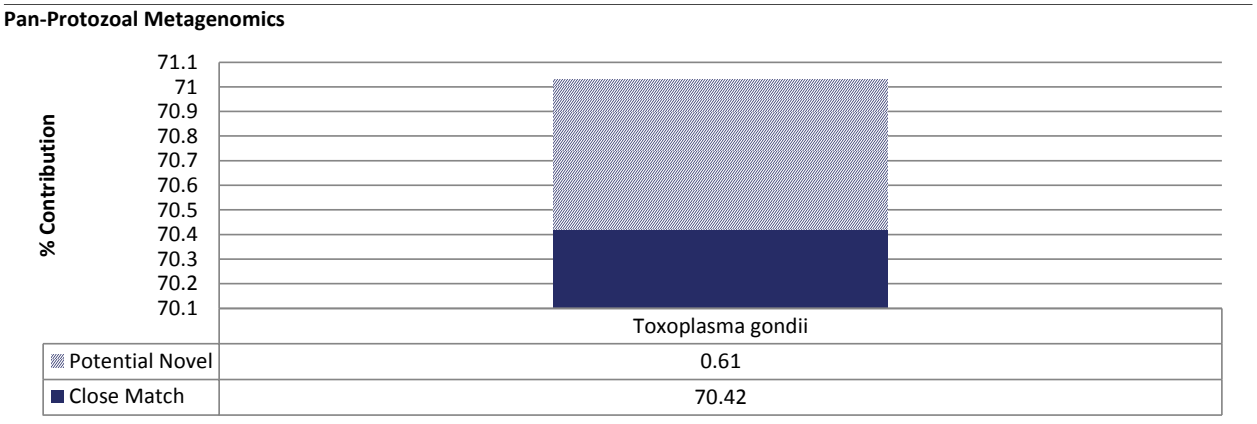
A 68-year-old Caucasian male machinist was in his usual state of health when he was involved in a motor vehicle accident. Within one month of the accident he developed a rapid onset of joint pain that was soon diagnosed as rheumatoid arthritis. The patient developed anemia, neutropenia, a variety of inflammatory markers, and a positive *Toxoplasma gondii* titer during examination (*Table 1*).

Table 1 - Test Results for Rapid Onset RA

Test	Result	Reference Range
ESR	49 mm/hr	0 to 22 mm/hr
Rheumatoid Factor	746 u/mL	< 14 u/mL
anti-CCP	> 500 u/mL	20 u/mL
T. gondii IgM	9.5 IU/mL	< 9 IU/mL
T. gondii IgG	341 IU/mL	< 9 IU/mL

Interestingly, *T. gondii* has been implicated as a causative agent in rheumatoid arthritis (11, 12). PCR confirmation of active *Toxoplasma gondii* was not successful by a national reference laboratory. However, sequencing by RIDI™ resulted in a positive detection of *T. gondii* (*Figure 4*) consistent with active infection or convalescence. While the patient was recalcitrant to traditional therapies, the rapid onset rheumatoid arthritis was finally controlled using a 4-drug rheumatoid regimen of methotrexate, prednisone, leflunomide, and rituximab. These therapies are particularly of interest as immunosuppressive therapy is known to contribute to Toxoplasmosis (13-15). When the patient was evaluated last, he was requesting clearance to return to work as a machinist.

Of note, NGS detected the presence of a strain of *T. gondii* that was undetectable by traditional targeted PCR tests. The sequencing findings are supported by the elevated *T. gondii* serology titers and demonstrates the value of NGS for the detection of novel strains or variants. The use of NGS for infectious diseases in complex and multifactorial patient populations may prove useful as to avoid potentially life-threatening complications between rheumatologic therapies and infections.



Complete Significant Contribution

Species Name	Close Match	Potential Novel	Total Percent	Match Count	Novel Count	Total Count
Toxoplasma gondii	70.42	0.61	71.03	1847	16	1863

Figure 4. Eukaryotic DNA Sequencing Results. NGS results indicates the presence of a *Toxoplasma gondii* strain in the peripheral blood from a patient with rheumatoid arthritis.

#3 Osteoarthritis

A 63-year-old Caucasian male contractor presented with a major complaint of global fatigue, joint pain, and a formal diagnosis of long-standing osteoarthritis. The patient had undergone numerous surgeries for severe degenerative disease due to the osteoarthritis. The patient's CRP (C-reactive protein) was 15.1 mg/L and hsCRP (high sensitivity C-reactive protein) was 13.95 mg/L. A vector-borne disease screen, including a Western Blot for *Borrelia burgdorferi*, IgG and IgM antibodies to *Bartonella henselae/quintana, Babesia microti, Ehrlichia chaffeensis, Anaplasma phagocytophilum, Rickettsia rickettsii/typhi*, and additionally *Toxoplasma gondii*, were negative. The patient was empirically placed on a course of doxycycline 100 mg b.i.d. and the patient exhibited gradual improvement over time. Staining of his peripheral blood using a fluorescent DNA stain revealed a complex of eukaryotic fungal conidia, hyphae and biofilm matrix, NGS revealed and confirmed abundant *Saccharomyces cerevisiae* in the peripheral blood (*Figure 5 and 6*). Patient felt symptomatic improvement enough on doxycycline that he declined the addition of an antifungal.

The underlying etiology of osteoarthritis has not been established, but new theories supported by MRI blood flow data suggests that a vascular obstructive process may play a role (16, 17). The vascular obstructive process is well documented in the scenario of CCSVI (Chronic Cerebral Spinal Venous Insufficiency) and venous obstructive disease in Multiple Sclerosis as popularized by Zamboni (18). Our study of carotid vascular debris and coronary plaque displays a rich population of eukaryotes (19). This suggests a role for previously undiagnosed microbes in the ischemic process. Our hypothesis is that these eukaryotic microbes may play a role in promoting or contributing to generalized vascular obstructive disease reducing overall flow; therefore, causing bone degeneration. This case provides evidence that *Saccharomyces* is present in an osteoarthritic patient and could potentially cause flow disruption in the bone vasculature causing degeneration. *S. cerevisiae* is implicated in many diseases and can result in sepsis (20-22). Elevated serologies against ASCA (anti-*Saccharomyces cerevisiae* auto-antibodies) and elevated serum IgA against *Saccharomyces cerevisiae* mannan are frequently observed in RA patients (23, 24). Doxycycline has been demonstrated to be an antifungal/ antibiofilm agent in *Candida albicans* and possibly has similar activity with *S. cerevisiae* (25, 26).

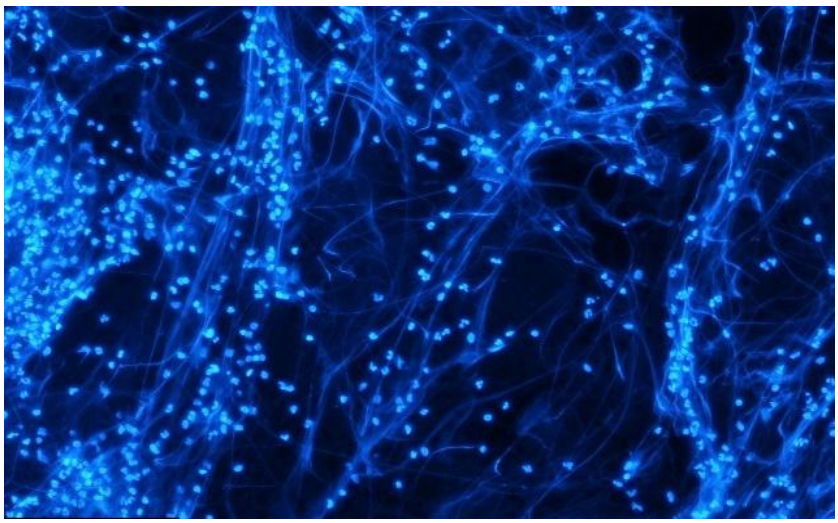
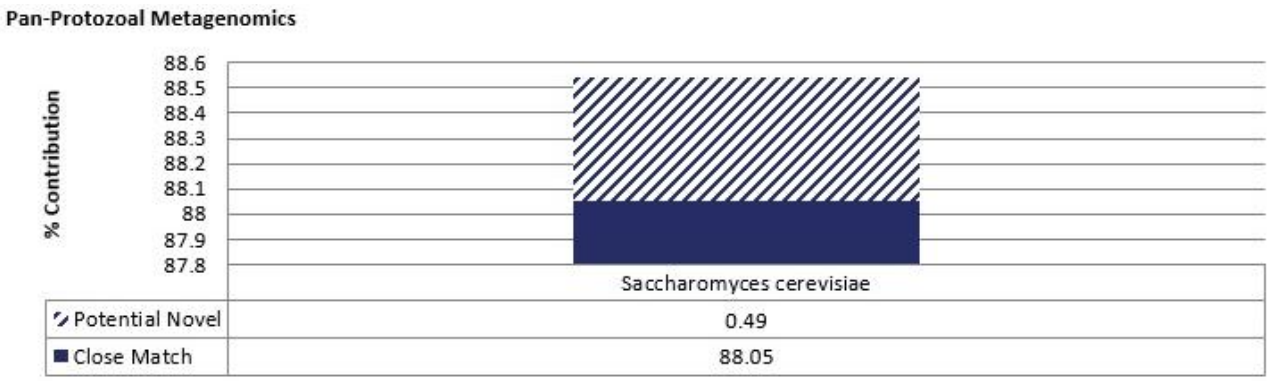


Figure 5. Fluorescence Microscopy of Wet Mount peripheral blood 400x, from a patient with osteoarthritis displaying fungal conidia, hyphae and biofilm matrix.



Complete Significant Contribution

Species Name	Close Match	Potential Novel	Total Percent	Match Count	Novel Count	Total Count
Saccharomyces cerevisiae	88.05	0.49	88.54	2505	14	2519

Figure 6. Eukaryotic DNA Sequencing Results. *Saccharomyces cerevisiae* was detected in a patient with osteoarthritis with a significant portion of reads with close sequence homology to the known reference standard.

#4 Chronic Fatigue Syndrome (CFS)

A 28-year-old Caucasian male presented with long standing debilitating fatigue, arthralgia, and myalgia with additional complaints of gastritis, jaundice, polyurea, headaches, irritable bowel syndrome, and iritis; all of which closely fit the Fukuda criteria for CFS(27). He had extensive travel to India, Kashmir, and Ecuador. Past vector-borne disease and infectious disease lab studies were inconclusive. In the past, the patient had been on multiple anthelminthic, antibiotic, and antimalarial medications. The patient had exhibited improvement in the past on a course of praziquantel. The patient also responded to an extended course of doxycycline, 100 mg b.i.d. but not as robustly as praziquantel. Fluorescent DNA stains illuminated the presence of putative eukaryotic communities in the peripheral blood (*Figure 7*). Next-Generation DNA sequencing targeting eukaryotes revealed the presence of a fungi most closely related to *Funneliformis mosseae*, i.e. 'best match' (*Figure 8*). The patient was treated with the addition of terbinafine, 200 mg. q.d. and after 6 months of therapy was asymptomatic. At 8 months, a recurrence of his symptoms occurred, and the patient was restarted on a terbinafine and doxycycline regimen.

Sequences with the closest match to *F. mosseae* are a common finding in the blood of chronically ill patients tested in our laboratory. In this case, there is significant variability in the percent identity to the reference strain. *F. mosseae* is a common arbuscular mycorrhizal fungus and a plant symbiont as it serves to extract nutrients from the soil, reduces heavy metals from entering plant roots, and is agriculturally important as it improves crop yields (28-32). *F. mosseae* exhibits significant genetic diversity and was formerly named *Glomus mosseae*. (33, 34). This fungi was the only microbe detected in all three cases of coronary plaque (16,19), and was a common finding in CFS patients and in some normal control patients [15, Ellis, J et. al, 2017, submitted]. The response this patient had using a combination of doxycycline and terbinafine, suggests clinical validity of NGS diagnosis. The patient's relapse suggests resistance to selected therapeutic agents.

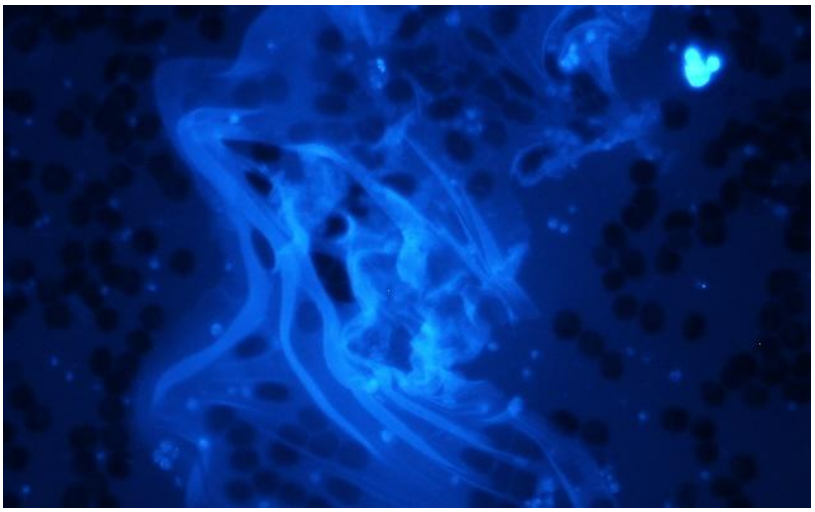
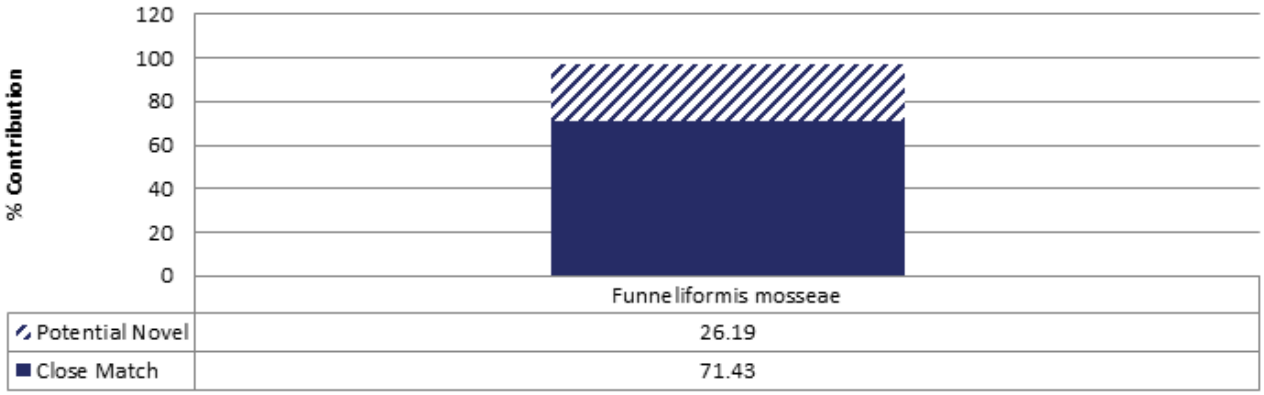


Figure 7. Fluorescence Microscopy of Wet Mount peripheral blood 1,000x, from a patient with CFS. Eukaryotic cells can be seen embedded in a DNA rich matrix.

Pan-Eukaryotic (Protozoa / Fungi) DNA Sequencing



Complete Significant Contribution

Species Name	Close Match	Potential Novel	Total Percent	Match Count	Novel Count	Total Count
Funneliformis mosseae (u)	71.43	26.19	97.62	300	110	410

(u) - Organism of unknown clinical significance.

Figure 8. Eukaryotic DNA Sequencing Results. *Funneliformis mosseae* variant was detected in a patient with CFS with a varying degrees of sequence homology to the reference standard. A non-insignificant portion (26.19%) of the detected sequences diverged more than 2% from the reference standard.

Interpretation of NGS RIDI™ results:

The results of an NGS run using RIDI™ are depicted as a bargraph with the closest match organism or organisms listed at the bottom of the bargraph (*Figure 3-5, and 8*). The scale for the genetic match rises to 100% of the total population. Taxonomically, if the sequences derived are within 2% sequence identity of the index organism then it is considered a match. Sequences diverging more than 2% of sequence identity are listed in hatch marks and closely correlate to organism divergence. In case #1 (*Figure 2*), the bars without hatch marks indicate the sequence read match is nearly

identical between the detected and reference organisms. In case #2 with rheumatoid arthritis (*Figure 3*), the close match (within 2% sequence identity of the index sequence) is 70.03% of the population of sequences detected. Divergent sequences represent 0.61% of the total sequence read population. The additional sequences in this run were non-diagnostic, as they do not meet result thresholds. In case #3 with osteoarthritis (*Figure 5*), 88.05% of the reads closely match the reference sequence with 0.49% of the reads diverging from the reference sequence. In this case, 12% of the sequence reads are considered non-diagnostic as they do not meet control thresholds. In the CFS case #4 (*Figure 8*) 71.43% of the detected sequences were closely matching the reference, while 26.19% of the sequences were more divergent from the reference organism. The contrast between each of the cases (Figure 4, 5, and 8) is significant with low novel sequence contribution in cases 2 and 3 compared to the significant contribution of novel sequences found in case #4, (*Figure 8*) diverging from the reference sequence for *F. mosseae* by 2% or more. The total sequence count results represent the relative amount of sequences that roughly correlate to the robustness of the sequencing results and by extension the number of organism's present. Generally, results with thousands of sequence reads are abundant and significant. The report attempts to simply represent the taxonomic diversity, but preserving the complexity of the findings. A clinician may ignore the contribution of potentially divergent or novel organisms and may simply rely on the genus and species-level identification for general therapeutic guidance in these cases. Efforts to simplify the report for the clinician are ongoing, but it is important to relay to the clinician the genetic divergence from the reference strains which could explain unexpected symptoms, clinical presentation, and/or drug resistance.

General Discussion

Now that NGS systems are available, it has become an incredible tool for the rapid and accurate diagnosis of known and well characterized microbes in addition to novel organisms. It is important to understand the RIDI™ system provides best match and is only as good as the database it uses; thus, the use of the NCBI database as a foundation. All databases are undergoing constant taxonomic revisions. As an example, reclassification of *Babesia microti* to *Theileria microti* has been proposed (35, 36). The primary concern with microbial detection by classic methodologies is the detection of uncultivable, novel, and variant organisms as well as the temporal delay in diagnosis. NGS has the potential of solving the mystery of chronic disease, especially inflammatory disease. Assays using standard or multiplex PCR are usually narrowly restricted, missing both novel and variant species. NGS microbial detection will be useful for organisms where testing is nonexistent or to strengthen identification by other methodologies. Additionally, NGS is a profoundly powerful tool in detecting unusual microbes and especially important for the categorization of new sequence information from a constellation of novel bacteria, fungi, protozoa, and algae.

The use of broad primers and an amplicon sequencing strategy allows for dramatically better test specificity and an improved ability to identify and discriminate amidst the background of human DNA in human samples. A recent publication describes the improved quality of amplicon sequencing compared to untargeted sequencing in respect to stool microbiome (37).

While bacterial and viral etiologies have been studied extensively as potential causation for chronic illness, the role eukaryotes play in these diseases have not been extensively investigated. Based on our last few years of clinical laboratory experience with NGS analysis, it is possible

that biofilm forming eukaryotes are playing a role in chronic disease processes such as cardiovascular disease, chronic fatigue syndrome, fibromyalgia, osteoarthritis, autoimmune disease, gastritis, and interstitial cystitis. NGS has obvious applications in the acutely ill where timely and accurate diagnosis is essential. The use of NGS for more difficult and chronic cases may greatly enhance favorable clinical outcome.

One of the drawbacks for NGS is the expense and relative novelty, these barriers to adoption in the clinical setting will dissipate as technology advances and physician education occurs. Legacy test technologies such as microscopy, culture, serologies, and more recently PCR have proven of great value, but NGS offers much more and may supplant some of these techniques. A recent viewpoint article discusses the great potential of NGS with heightened sensitivity but raises concerns about the potential of overtreatment from overcalling positive results and the need for ”Diagnostic Stewardship” (38). The RIDI™ system uses sophisticated bio-informatics to insure meaningful diagnostic calls and reduces the risk of false calls, distinguishing real infection vs. background. Also, a clinician will and should always be in the information and treatment loop combining clinical experience with heightened diagnostic capability armed with NGS results. As clinicians, we must not fall into a pattern of prejudgment on the role of novel and environmental microbes, especially, highlighted in the case report of osteoarthritis where *S. cerevisiae* is now implicated as having pathogenic potential.

Since the capability and the clinical validity of NGS has been established, the question that remains is how could a treating clinician treat these variant and novel organisms in association with disease and a symptomatic patient? It is logical and reasonable to treat as we would the closest relative of which we have clinical experience. Treatment of bacterial infections, microbial drug resistance /sensitivity gene markers are in the process of being developed and should be available in the future to help make the correct antibiotic choices. Fry Laboratories initiated a study whereby outcome surveil-lance can be correlated with disease genotyping. In the future, this database, registry, and others like it may provide better treatment strategies. The RIDI™ system is currently validated for the detection of bacteria, archaea, protozoa, fungi, and algae. Global amplicon based viral detection has gone through proof of concept and is undergoing further development.

Summary

NGS technologies may take some time for adoption; however, older molecular technologies, such as PCR, have paved the way. Clinical understanding of the wide array of potential microbes is paramount in the accurate diagnosis of disease. The RIDI™ system is an example of an integrated clinical system capable of providing accurate clinical results in a timely manner. The cost of sequencing will decrease as the ability to reliably multiplex samples expands, automation of systems, faster chemistries and new technology platforms enter the marketplace. The ability to accurately and rapidly diagnose infectious disease and develop a better understanding of the microbial environment can change our perspective on acute and chronic illnesses. NGS may one day result in improved selection of therapeutics, while encouraging the creation of superior therapeutics and potentially leading to disease cures. The value of this universal unbiased approach, facilitated by the RIDI™ system, allows for a non-discriminatory look at the infectious disease setting of the microbiome and pathogenic potential in humans.

Declarations: Dr. Stephen Fry is the owner of Fry Laboratories and holds patents on the RIDI™ system.

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Abbreviations:

NGS-next generation sequencing

PCR-polymerase chain reaction

NCBI- National Center for Biotechnology Information

RIDI- Rapid Infectious Disease Identification System

ISP- ion sphere particles

ISFET- ion sensitive fixed effect transistor

CRP-C- Reactive Protein

hsCRP-high sensitivity CRP

CCSVI-Chronic Cerebrospinal Venous insufficiency

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