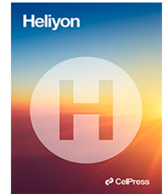
Heliyon 10 (2024) e34538

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

ICU care through NGS – Based identification of infectious agents: A comparative study

Aishwariya Ramanathan [a,*], Anusuya Marimuthu [a], Lalanika M. Abayasekara [b], Thurga Jeyaratnam [b], Vishvanath Chandrasekharan [b], Jennifer Perera [b], Venkatasubramanian Ramasubramanian [c], Nandini Sethuraman [c], Sumudu Samarasinghe [b], Varuna Peiris [b], Vaz S. Gnanam [a,b]

[a] *Micro Genomics India Pvt Ltd, 16, Wallace Garden First street, Thousand lights, Chennai, 600006, India*
[b] *Credence Genomics, No 12 3/2, Sunethredevi road, Kohuwela, Nugegoda, 10250, Sri Lanka*
[c] *Apollo Hospitals, Greams road, Thousand lights, Chennai, 600006, India*

ARTICLE INFO

Keywords:
Sepsis
Pan bacterial identification
Pan fungal identification
16s rRNA sequencing
ITS sequencing
Next generation sequencing
Metagenomics

ABSTRACT

Background: Sepsis claims 1 in 5 lives annually as per global statistics. Sepsis incidence in recent studies represents at least 35 % of all ICU admissions and has a high mortality rate, especially in the presence of co-existing morbidities. The challenge has been to accurately diagnose the causative organism, considering factors such as possible polymicrobial infections, commensals and environmental contaminants. Legacy techniques such as culture, automated culture systems or even newer species-specific PCR or film array these challenges difficult to overcome. The Bactfast® and Fungifast® assays along with the integrated workflow is based on next generation sequencing and have the ability to demarcate infecting pathogen from contamination and commensal. The unique ability to pinpoint the exact pathogen, considering the commensal and contamination in a variety of samples, with an extremely high sensitivity could lead it to be a tool of diagnostic choice for non-resolving ICU sepsis due to its comprehensive coverage and speed. The aim of this study was to evaluate the use of Bactfast® and Fungifast® as a last mile diagnostic tool in a ICU setting.

Method: This study was carried out considering access to four intensive care units (ICU). Legacy testing, mostly done on culture, was conducted at the various integrated microbiology facilities of the hospitals where the ICUs were located, in Chennai, India. NABL accredited laboratory Micro Genomics (India) Pvt Ltd, was established as the central processing facility for next generation sequencing to run the Bactfast® and Fungifast® assay. Co-relation of results for 490 samples was done retrospectively by a multi-disciplinary team of consultants which comprised of microbiologists, and infectious disease physicians.

Result: The diagnostic workflow established with the Bactfast® assay provided a sensitivity of 94.1 % and specificity of 86.6 %. Identification of pathogens in Bactfast® was better when compared to the data published in 2017, as reflected by positive co-relation with clinical confirmation. Although the Fungifast® specificity was high, at 99.4 %, only 12 samples were positive on fungal culture out of 490 samples. Therefore, it was concluded a further study for fungi based on multiple technologies with more true positive samples is required to evaluate the test.

* Corresponding author.
E-mail address: aishwariya@microgenomics.in (A. Ramanathan).

https://doi.org/10.1016/j.heliyon.2024.e34538
Received 27 March 2024; Received in revised form 5 July 2024; Accepted 11 July 2024
2405-8440/© 2024 Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Conclusion: Bactfast® can identify pathogens in a sample without any bias. Its introduction as diagnostic modality in life threatening ICU sepsis could reduce mortality and morbidity. Although the initial results of Fungifast® are encouraging a further research is required for more information on test sensitivity.

1. Introduction

Diagnostic testing plays a crucial role for identification of pathogens especially in patients with sepsis in the ICU. Sepsis and septic shock account for 35 % of all ICU admissions [1]. A number of conventional techniques are used to identify pathogens from clinical samples, the most common being culture based tests, coupled with biochemical assays and followed by susceptibility testing if applicable. However, the presence of commensals, environmental contamination, fastidious and competitive nature of microorganisms, along with the inherent limitations of culture can lead to inconclusive results [2–4]. Delay in culture turnaround times compounded with the above, calls for novel technologies to be implemented in pathogen detection [5].

Since the COVID epidemic clinical microbiology has seen an increase in the adoption of molecular diagnostics for pathogen detection from a clinical sample [6]. However, molecular diagnostic methods such as PCR and film array have limitations because of their inherent inability to cover a wide variety of organisms, hence making it unsuitable for use as an agnostic diagnostic test. An agnostic diagnostic is especially important in an ICU setting where speed and accuracy are important for treatment.

1.1. Metagenomics via next generation sequencing

Molecular diagnostic techniques using a universal broad range PCR, targeting 16S and ITS regions, can be used to identify bacteria and fungi respectively [7–9]. In a study published in 2017, Abayasekara et al. described the development and testing of a diagnostic test, which is now trademarked as Bactfast® and Fungifast®. These assays use customized primers to amplify partial 16s rRNA and ITS regions from bacteria and fungi, and allow multiplexing of multiple samples on a single sequencing run [10]. The sequenced data is analyzed using a proprietary bioinformatics pipeline to identify the pathogenic organisms while filtering out commensal and contaminant information within the sample. These assays are processed direct from sample and are applicable to a wide variety, including tissue. This study further validates Bactfast® & Fungifast® assays for clinical application, across multiple sample types, specifically within an ICU setting.

2. Methods

2.1. Study design

This was a real time prospective study, including four hospitals from Chennai India; Apollo hospitals Greams road, Apollo first med, Apollo multispeciality Vanagaram and Apollo hospitals OMR.

The study period was from January 2018 to December 2019. Specimens were taken from clinical sites including blood, body fluids from sterile sites (including CSF, joint fluid, pleural fluid, pericardial, peritoneal fluid, intraabdominal collections), pus (by needle aspiration) and tissues obtained intraoperatively (including sterile biopsy, bone, heart valves etc). The samples were subjected to conventional culture for bacteria and fungi at the hospital's mentioned and their self-contained microbiology facilities. In parallel each sample was processed for Bactfast® & Fungifast® at the Micro Genomics India Pvt Ltd facility. The results were obtained in 36 h and submitted to the clinical panel for comparison retrospectively.

2.2. Sample collection

For blood samples alone, informed consent was obtained from patients for inclusion in to the study. 2 ml of blood was collected into EDTA vials subsequent to 10 ml collection for blood cultures.

2.3. Sample collection from sterile sites

Clinical samples from ICU wards were collected under aseptic conditions and transported to Department of Microbiology of the relevant hospital. Upon receipt, minimum of 1 ml for fluid samples and a minimum of 25 mg of tissue samples were aliquoted (inside a biosafety cabinet), sealed and transported in a sample carrier at 4 °C to Micro Genomics laboratory for processing. The remaining sample was taken for conventional culture testing.

2.4. Conventional culture based testing

Conventional testing included microscopy, bacterial or fungal cultures, identification by MALDI-TOF, (VITEK-MS, Biomerieux), VITEK-2 compact (Biomerieux), conventional biochemical reactions or fungal morphology as applicable.

3. NGS testing

3.1. DNA extraction

All samples were taken for DNA extraction using Bactfast®/Fungifast® custom extraction protocols using the QIAmp DNA mini kit (Qiagen). Protocol for DNA extraction is available in Additional File 1.

3.2. Library preparation

The extracted bacterial/fungal DNA was subjected to PCR using the Bactfast® and Fungifast® protocols, according to the manufacturer's instructions [10]. The amplified products were visualized on a 1 % agarose (SeaKem LE Agarose) and purified using 1X NucleoMag^{R}(Machery-Nagel).

3.3. Template preparation and sequencing

All the samples were sequenced on the Ion Torrent S5 XL system, using the OneTouch2 template preparation protocol. The samples were pooled in batches of either 20 or 60 for the 520 or 530 chip respectively, and sequenced according to manufacturer's instructions for 400bp sequencing.

3.4. Contamination depletion in analysis

A negative extraction control (EC) at DNA extraction step & NTC at PCR amplification was run for each batch. In case of contamination in the EC and NTC, these controls were sequenced and the contaminants were depleted from the sample output where resampling was not possible. In cases where sufficient sample was remaining, the workflow was repeated.

3.5. Bioinformatics analysis

Unaligned Binary alignment map (UBAM-) with reads that had > Q20 score, were taken for analysis and trimmed to remove adaptor sequences. Quality control parameters (Phred Quality Score cut off and minimum read length) for all the sequenced data were checked. The average number of reads per sample ranged from 75,000 to 100,000.

Two pipelines were used in parallel to check for reproducible outputs based on the sequence data. For the primary pipeline, alignment was conducted using a MegaBLAST (blast - 2.5.0) tool from NCBI and a modified MySQL database application used to identify known accession numbers with taxonomic representation based on Refseq sequences for 16S rRNA & ITS regions. The secondary pipeline was linked to similar accession data from Refseq using the kraken2 algorithm. Reproducibility was compared on both algorithms for consistency.

3.6. Determining environmental bacteria and fungi diversity representation

To increase the accuracy of pathogen identification, a baseline environmental sampling was performed prior to study initiation. Swabs from beds, tables, floors, walls and stands were taken in sterile 1X PBS (Phosphate buffer saline) from all the ICU wards of the participating hospitals to monitor possible environmental contamination during the sample collection. Based on the incidence of the microbial flora at each ICU, a total of 6 baseline filter files were built. The environmental organisms were grouped based on operational taxonomic units (OTU) using a closed reference method [11]. These files were trained and integrated into the pipeline to filter contamination if applicable and increase sensitivity to pathogenic organisms from the final output. The environmental baseline filter files are available as Additional file 2–13.

3.7. Identification of pathogen based on threshold representation

On identification of the various species, the pathogen is established through an algorithm at threshold, and commensal or contamination demarcated according to the site of sample collection based on machine learning. The machine learning algorithm was trained using over 1200 clinical samples over a period of 3 years (unpublished data). Threshold representation (i.e. expected normal relative abundance of taxa) for each sample type and site of collection, is based on average representation across all trained samples.

For all samples, following classification in diversity representation, pathogen presence with respect to contributing species had a cross sample average of 10 % on aggregated data sets. Hence the threshold for the identification of a species that is pathogenic was established as 10 % on the pipeline. In cases where only commensals and/or contaminants are found, the samples are deemed as negative or clinically insignificant.

3.8. Clinical categorization of results

Results from NGS and culture were scrutinized by a panel of clinicians. The panel comprised of infectious disease specialists, physicians, and medical microbiologists. In cases where NGS and culture results were mismatched, a collective decision on results was

made based on the clinical history, site of infection and the symptoms, along with post treatment resolution.

Culture positive NGS positive (CPNP): Culture positive samples with the same pathogen identified by NGS, with or without additional species.

Culture positive NGS alternative flora (CPNA); Culture positive samples with different pathogens identified via NGS

Culture positive NGS negative (CPNN): Culture positive samples which were negative by NGS were categorized as CPNN

Culture negative NGS negative (CNNN): Samples with both culture and PCR results negative

Culture negative but clinically compatible (CNCC): Culture negative samples but NGS positive with clinically compatible results

Culture negative but clinically incompatible (CNCI): Culture negative samples but NGS positive with clinically incompatible results

3.9. *Evaluation of clinical compatibility/incompatibility*

When the culture results differed from the reported pathogen in NGS results, categorization was made based on clinical history, epidemiology and ancillary investigations. When the culture was negative but NGS identified pathogenic organisms, observation of the patient was initiated for 2–3 days (mean 2.5 days) to evaluate the patient response and assess whether the pathogen detected in NGS was clinically compatible.

4. Results

A total of 490 samples were received for microbial culture (bacteria or fungi) and Bactfast®/Fungifast® during the study period. Breakdown of samples tested in this study is described in Table 1. **Additional file 14** contains the detailed culture and NGS results for the 490 samples.

Samples were considered positive for bacteria or fungi if they showed bands >400bp and >200 bp respectively and were designated as PCR positive. The number of sample designated in each category for Bactfast® and Fungifast® results were shown in Table 2.

4.1. *Bactfast® concordance results*

A total of 16 samples from the 111 PCR positive were reported as mixed infections from bacterial culture which was also reflected in the Bactfast® results. Additionally, anerobic organisms (*Bacteriodes* spp) were detected by NGS in 6 samples along with the pathogen reported in the bacterial culture. These samples were designated as CPNP.

Bactfast® was positive for 50/372 bacterial culture negative samples. Out of 50, 47 samples were found to be correlating with the clinical history by the panel of clinicians based on the criteria mentioned above and were designated as CNCC.

4.2. *Bactfast® discordance results*

7/118 samples showed different organisms on Bactfast® analysis versus bacterial culture results and were designated as CPNA under discordance. 3 Bactfast® positive in the culture negative samples were incompatible with clinical findings and categorized as CNCI.

4.3. *Fungifast® concordance results*

A total of 7/12 fungal culture positives were positive for Fungifast® PCR with the same organism reported in fungal culture results. All these samples were categorized as CPNP. A total of 475/478 fungal culture negative samples were negative on Fungifast®. These samples were included in CNNN category. Fungifast® was positive for 3/478 fungal culture negative samples. Of these, 2 samples were determined to be correlating clinically (CNCC).

4.4. *Fungifast® discordance results*

A total of 5/12 fungal culture positive samples were negative on Fungifast® PCR and were designated as CPNN. One culture negative sample was positive in Fungifast® but was found to be clinically incompatible (CNCI).

Table 1
Types and number of clinical specimens analyzed.

Specimen type	No of specimens
Blood	183(37.4 %)
Body fluid	204(41.6 %)
Tissue	91(18.6 %)
Pus	12(2.4 %)
Total	**490**

Table 2
Total number of samples designated under each category according to comparison of NGS results with culture and clinical co-relation.

Concordant Bactfast® and Fungifast® NGS results			
Culture results	Catogories	Bactfast®	Fungifast®
Culture positive	Culture Positive NGS Positive (CPNP)	111	7
Culture negative	Culture Positive NGS Negative (CNNN)	322	475
Discordant Bactfast® and Fungifast® NGS results			
Culture results	Catogories	Bactfast®	Fungifast®
Culture positive	Culture Positive NGS alternative flora (CPNA)	7	0
	Culture Positive NGS Negative (CPNN)	0	5
Culture negative	Culture Negative Clinically Compatible (CNCC)	47	2
	Culture Negative Clinically Incompatible (CNCI)	3	1

4.4.1. *Analysis of NGS and culture results*

Culture positive and culture negative samples designated as concordant and discordant are tabulated based on the sample type in Table 3. As shown in Table 3 Bactfast® results, 98.9 % of the tissue samples and all the pus samples were concordant with culture results. However, only 89.1 % of the blood samples, 82.4 % of the body fluids were concordant when compared with culture. From the discordant results 7.1 % of blood samples, 16.2 % of body fluid and 1.1 % of tissue results were considered clinically compatible, giving a total of 96.2.% of blood samples, 100 % of tissue samples and 98.6 % of body fluid as concordant

4.4.2. *Specificity and sensitivity for Bactfast® and Fungifast®*

The large sample size used in this study, is sufficient to provide statistically significant results on specificity and sensitivity of Bactfast® in sterile clinical samples. However, while there is a large sample size for fungal negative samples, only 12 fungal positive culture samples were detected. The specificity and sensitivity were calculated using the formulas as described below and the sample numbers were shown in Fig. 1:

Sensitivity = True Positive/(True Positive + False Negative).

Specificity = True Negative/(True Negative + False Positive).

This study demonstrated that Bactfast® has a sensitivity of 94.1 % and specificity of 86.6 % However, when considering clinical compatibility the sensitivity and specificity goes up to 95.8 % and 99.8 % respectively.

While specificity of Fungifast® is 99.4 %, there were too few fungal true positive samples to conclusively assess Fungifast® sensitivity.

5. Discussion

Due to the limited sensitivity and narrow spectrum of coverage of conventional molecular techniques such as species specific PCR, most ICUs do not implement these assays in routine investigations and instead rely on conventional culture based diagnostic testing. However, there are drawbacks to relying solely on this test, specifically where unusual or slow growing microbes are present [12].

Table 3
Sample wise breakdown of culture positive, culture negative, concordance and discordant results from Bactfast® and Fungifast®.

Bactfast®results					
	Bacterial Culture positive		Bacterial Culture negative		
	Concordance	Discordance	Concordance	Discordance	
	CPNP	CPNA	CNNN	CNCC	CNCI
Blood	20	5	144	13	2
Body fluid	39	2	129	33	1
Tissue	45	–	44	1	–
Pus	7	–	5	–	–
Total	111	7	322	47	3
Fungifast® results					
	Fungal culture positive		Fungal culture negative		
	Concordance	Discordance	Concordance	Discordance	
	CPNP	CPNN	CNNN	CNCC	CNCI
Blood	2	4	178		–
Body fluid	3	1	197	2	1
Tissue	2	–	88	–	–
Pus	–	–	12	–	–
Total	7	5	475	2	1

	Culture				Culture		
Bactfast®		Positive	Negative	Bactfast®		Positive	Negative
	Positive	111(TP)	50(FP)		Positive	158(TP)	3(FP)
	Negative	7(FN)	322(TN)		Negative	7(FN)	322(TN)
Without clinical correlation				Clinical correlation			
Sensitivity	94.10%			Sensitivity	95.80%		
Specificity	86.60%			Specificity	99.10%		

Fig. 1. Sensitivity and specificity for Bactfast®

As demonstrated previously, clinical metagenomics is a promising field for application in clinical diagnostics [10]. However, as a relatively new field, it has to be assessed in conjunction with conventional and wide-spread techniques currently in use. While targeted molecular diagnostic testing for infectious pathogens is more wide spread; when negative, the physician will subsequently proceed through a list of different tests to pinpoint the correct diagnosis. Therefore, a universal test for the diagnosis of infectious organisms is more useful in a clinical setting because it eliminates the guess work from the diagnostic process.

In the previous study [10], it was clear that more species were detected in the Bactfast® work flow vs culture. However, it also demonstrated that clinical history is required for better assessment of the sensitivity and specificity of the tests. Therefore, clinical co-relation was introduced as part of this study to provide better resolution on clinical application.

Unlike conventional 16S or ITS1 metagenomics, Bactfast® and Fungifast® are specifically designed for identification of pathogens and allows depletion of environmental flora, and lab contaminants from final analysis output.

5.1. *Diversity representation for pathogen identification and depletion of commensals/contaminants*

The bioinformatics analysis used to deliver the results of this study is unique in its ability to define pathogens as well as commensals and deplete environmental contaminants. For each analysis, depending on the sample and site, the pipeline would run an algorithm to consider the diversity representation along with threshold representation.

Alpha diversity that is seen within the sample would show a higher richness of speciation in non-sterile vs sterile samples. In a sterile sample, alpha diversity would show less species diversity and when trained against closed reference OTUs, there is a clear difference versus OTUs representing contamination or commensals.

In a non-sterile sample, the pipeline will consider beta diversity based on similar samples that have been used previously to train the machine learning algorithm. The algorithm is able to demarcate the species across the sample and eliminate commensal information. The proprietary algorithm would aggregate the information across the sample and the closed reference OTUs, and bucket the sample as contaminated or not.

Pathogen identification in sterile samples is fairly straightforward as the presence of any species not seen in the trained data set (as normal flora) or not matching the environmental baseline would be flagged as pathogen. On average pathogenic species will be present in very high abundance in sterile samples making pathogen identification relatively easy.

For non-sterile samples following classification in diversity representation, the pathogen would be identified with respect to the contributing species, at threshold representation. Pathogens in non-sterile samples have a cross sample average of 10 % in all trained data sets based on the output of over 1200 clinical samples. Therefore, the threshold for identification of a species that is pathogenic was established as 10 % on the pipeline. This also applies in cases of polymicrobial infection, where more than one species is identified as the pathogen. In such cases the information can be passed to a clinician for final review and co-relation of clinical symptoms for infection.

This also has an impact when considering competition from contaminants (introduced during sample collection) in samples that are difficult to collect such as CSF. This algorithm would ensure that commensal or colonizers for that site were accounted for and contaminants could also be removed where applicable.

5.2. *Breakdown of Bactfast® & Fungifast® vs. culture results*

An overall analysis of the results from NGS and culture, revealed that all bacterial culture positive samples were also Bactfast® positive, though this trend was not observed with fungal culture.

6. Culture positive results comparison

6.1. Bactfast®

The Bactfast® workflow detected bacterial DNA and successfully detected mixed infection which was corroborated by culture results. Additionally, anaerobic organisms were detected where the culture failed, increasing the accuracy of this test, when compared with bacterial culture. Furthermore, in 3 (MG/RID/249, MG/RID/261, MG/RID/264) tissue samples and 2 (MG/RID/387, MG/RID/415) body fluid samples, *Streptococcus* spp were detected along with the pathogen detected in culture sample, indicating the possibility of co-infection with these species. Bactfast® results showed the presence of *M. tuberculosis* in 2 (MG/RID/58 and MG/RID 281) samples which matched with the culture results. This demonstrates that Bactfast® can cover anaerobic and polymicrobial infections as well as slow growing and fastidious organisms within 36 h. However the viability of anerobic organisms identified cannot be assessed particularly considering that they were not grown in culture.

As shown in Table 4, a small number of bacterial culture positive samples (5.9 %) showed conflicting results with Bactfast® analysis. In sample MG/RID/289, skin flora (*S. epidermidis & Cutibacterium acnes)* was detected, which may have been introduced to the sample during collection and may have resulted in the actual pathogen being masked from detection. This is demonstrated in sample MG/RID/324 (pleural fluid), where *E. coli* (as detected in culture) was found in a very low abundance in Bacfast® result. In these cases, the requirement for stringent sample collection parameters is demonstrated. As skin flora or commensals are not typically expected in these samples and the algorithm is unable to deplete the contaminants to successfully identify the pathogen.

In the remaining five samples, different species are reported in culture vs Bactfast®. *Acinetobacter* spp from 1 sample and *Enterobacter* spp from 2 samples from culture vs *Stenotrophomonas maltophila* identified by Bactfast®, This pattern was observed in another two discordant samples, indicating there could be possibility of mis-identification in Bacfast® or culture. However, re-sampling was not possible in these cases due to time constraints and the pathogen could not be conclusively identified. Therefore these were designated as discordant results. The discordant results were observed only in blood and body fluid sample types, whereas Bactfast® has shown a high concordance in tissue and pus samples.

As both blood and body fluid samples generally require piercing the skin barrier, the discordant Bactfast® results show how critical sample collection parameters are to maintain the accuracy of the result, specially in cases of sterile samples.

6.2. Fungifast®

Table 5 shows the detailed breakdown on Fungifast® results for the conflicting samples. Positive sample co-relation was less straightforward in Fungifast®. While 58 % of the fungal culture positive samples were detected on Fungifast®, the rest were negative (5/12). Of the 5 fungal culture positives, 3 were positive for Bactfast® but negative for Fungifast®. As the DNA extraction protocol for Fungifast® does not specifically exclude the elution of bacterial DNA simultaneously, it is possible that a high bacterial DNA load would have affected the yield of fungal DNA and thereby lead to insufficient amplification.

4 of the fungal culture positive samples that were negative in Fungifast®, were blood samples which may be a result of the low input volume for Fungifast® workflow (1 ml) vs culture (5–10 ml). Further testing with increased input volume might help improve Fungifast® sensitivity. The small number of fungal culture positives (12/490) makes it difficult to conclude the statistical significance of discordant results.

Table 4
Detailed breakdown of Bactfast® discordant results vs culture Positive and negative samples.

Bacterial Culture Positive vs Bactfast® discordant results				
CPNA(Culture Positive NGS Alternative flora)				
SRN	Sample ID	Sample	NGS result	Culture results
1	MG/RID/289	Blood	*S. hemolyticus,* *C. acnes*	*E. cloacae*
2	MG/RID/290	Blood	*S. maltophila*	*Acinetobacter* spp
3	MG/RID/319	Blood	*E. anophelis,* *S. maltophila*	*Enterobacter* spp
4	MG/RID/320	Blood	*S. maltophilia*	*Enterobacter* spp
5	MG/RID/324	Pleural fluid	*P. melaninogenica,* *S. pneumoniae,* *E.coli*	*E. coli*
6	MG/RID/349	Blood	*P. putida*	*B. cepacia*
7	MG/RID/491	Pleural fluid	*Acinetobacter* spp	*E. faecalis*
Culture negative Bactfast® discordant results - CNCI				
SRN	Sample ID	Sample Type	Bactfast Results	Culture Results
1	MG/RID/317	Blood	*Clostridium* sp	Negative
2	MG/RID/365	Blood	*P. aeruginosa*	Negative
3	MG/RID/382	Peritoneal fluid	*A. baumanii.* *E. anopheles*	Negative

Table 5
Detailed breakdown of Fungifast® discordant results vs culture Positive and negative results.

Fungal Culture positive Fungifast® discordant results				
CPNN (Culture Positive NGS Negative)				
S no	Sample ID	Sample	NGS	Culture results
1	MG/RID/317	Blood	*Clostridium* spp	*C.auris*
2	MG/RID/318	Blood	Negative	*C. auris*
3	MG/RID/382	Peritoneal fluid	*A. baumanii,* *E. anopheles*	*S. cerevicae*
4	MG/RID/364	Blood	Negative	*Candida* spp
5	MG/RID/365	Blood	*P. aeruginosa*	*Candida* spp
Culture negative Fungifast® discordant results – CNCI(Culture negative clinically incompatible)				
1	MG/RID/500	CSF	*Candida* spp.	No growth

6.3. Culture negative results comparison

It is well established that PCR is more sensitive than culture [13,14]. As Bactfast® and Fungifast® are PCR based tests they are more likely to detect pathogens in culture negative samples.

From the total culture negative sample pool (n = 372), 50 Bactfast® samples were positive. Furthermore, clinical evaluation of these samples led to the clinical compatibility of NGS results established in 47/50. For example, in MG/RID/374 *M. tuberculosis* was detected, in Bactfast® workflow, whereas culture was negative but the same sample was positive on gene xpert. In MG/RID/372 Bactfast® detected *K. pneumoniae* and although the culture report was negative the patient had been previously diagnosed with *K. pneumoniae*.

3/50 Bactfast® results could not be correlated with clinical symptoms. The contamination control parameters explained above were applied to determine whether these results were due to contaminants. However, the results were not reflecting the environmental microbial profile and therefore were ruled out as environmental contaminants. As this discrepancy could not be resolved, these samples were considered discordant.

3 culture negative samples were positive for Fungifast®. Of these, 2 Fungifast® results correlated with the clinical symptoms which allowed the consideration of the Fungifast® results as clinically compatible. However, one Fungifast® result of a CSF sample was discordant as clinical co-relation and environmental baseline could not sufficiently explain the Fungifast® results.

6.4. Limitations of the tests

The findings in the discordant results shows that the presence of commensals in the sample skews the results even when a diversity representation threshold has been drawn. A limitation of these tests is that every sample generates a fixed amount of data in order to meet the turn around time and ensure that the test is cost effective. The introduction of environmental microbial contaminants can skew the results by competing for resources during amplification and sequencing, which is then reflected in the data output. Therefore, stringent sample collection methods need to be followed.

Additionally for the optimal application of Bactfast® or Fungifast®, clinical history containing site of collection and sample type would help delineate commensal contribution against a pathogen. As the system is trained on existing data, emerging pathogens such as noscomial infections or organisms that have been newly discovered would be a challenge for the algorithm and clinician input will be required. Another limitation would be in cases such as intestinal dysbiosis where diversity patterns change in disease or treatment and therefore determining the pathogen would require clinical intervention.

Furthermore, Bactfast® or Fungifast® assays are unable to identify the viability of the detected organisms. Molecular based techniques are typically unable to distinguish between live and dead organsims and as such must be prescribed with these limitations in mind. Finally Bactfast® or Fungifast® workflows rely on agarose gel electrophersis which can add to time and labour. This can be reduced by adapting more advanced techniques in library preparation however, these methods are likely to increase the cost of the test.

6.5. Limitations of the study

As culture is the standard application used for pathogen identification in the various healthcare units, this study only evaluates Bactfast® or Fungifast® tests against culture. Further confirmation with another form of testing (such as PCR or film array) would have allowed objective resolution of discordant samples but this was outside the scope of this study.

Second, considering next generation sequencing to be a highly sensitive testing method, ideally ad hoc sequencing of the same sample at another laboratory would have reinforced the workflow established. The main challenge here was to train and establish the assays at another site during the study period, as they are relatively new to the market. However, Micro Genomics India Pvt Ltd, where the NGS processing was carried out, has positive and negative pressure rooms to operate Pre and Post PCR workflows, regular decontamination procedures and intensive training in handling clinical metagenomics which reduced the risk of errors in the output.

Next the inability to re-run discordant samples for conclusive resolution either due to lack of volume, or inability to recollect a

sample due to patient movement or discharges, resulted in continued ambiguity. This is particularly evident in the few discordant results from Bactfast®.

Finally, clinical confirmation was retrospective (i.e. after patient response and treatment had been observed) which in cases of discordance especially relating to multiple etiologies meant that the clinical team could not draw on a universally accepted conclusion.

6.6. Comparison with other infectious disease diagnostic tests

Ever since the pandemic, infectious disease diagnostics has looked for comprehensive multi-pathogen identification for quick and accurate identification. PCR was popularized during the pandemic, followed by the introduction of syndromic panels on film array. However, neither technology has not been able to provide comprehensive coverage as a true agnostic test. Targeted amplification followed by metagenomic next generation sequencing has been used to evaluate abundances of microorganisms in multiple settings apart from healthcare [15,16]. However, Bactfast® and Fungifast® have established an agnostic test which can identify a pathogen in any sample while demarcating contaminants, and commensals in the same test. Further research on comparison of Bactfast® and Fungifast® vs other pan-infectious tests would establish a series of comprehensive use cases and scenarios where this technology can be applied.

7. Conclusion

Utilization of Bactfast® in an ICU setting has shown advantages of NGS based pathogen identification in sepsis. A comprehensive clinical history with site and sample information is important for establishing possible commensal, and contaminant competition. Bactfast® results can be generated within 36 h, and provides data on the full spectrum of microbial species within a sample including anaerobic, and slow growing organisms. This is especially important in cases where culture, and other testing methods may have limited sensitivity or provide ambiguous results as Bactfast® will still provide information on pathogen by taking advantage of comprehensive machine learning in demarcating commensal and contaminant data. This test can be performed directly on a clinical sample and has no limitations on sample type.

Fungifast® could not be comprehensively assessed on its full clinical diagnostic potential. As only 12 fungal culture positive samples were identified from the entire 490 study cohort, any conclusions on sensitivity cannot be considered statistically significant. Therefore, further research with true positive samples will be required to establish Fungifast®.

Ethics approval and consent to participate

Only left over clinical samples were used for the identification of bacterial and fungal DNA. Human DNA was not used in the test. For blood samples alone, where a sample collection was performed specifically for the Bactfast® & Fungifast® tests, written informed consent was obtained from patients through Apollo research and innovations team (AAHRP). For publishing the data, written informed consent was not required as patient data was anonymised, test results were assessed under blinded conditions and no images or other information that may identify the patient is published. Study protocol was submitted to AAHRP accredited Apollo research and innovations with Ref no; IEC-CS App no: AMH-001/02–18 and approved on March 21, 2018, by the institutional ethical committee.

Funding

This study is industry sponsored, with funding provided by Micro Genomics India Pvt Ltd, India and Credence Genomics Pvt. Ltd, Sri Lanka

Availability of data and materials

The FastQ files and result analysis files including kraken, mysql, kraken mysql comaparison, phylogeny and filter baseline files generated during the study are available in the Mega. io repository,[https://mega.nz/file/Q7MWSBgQ#GJszZRbbjETf6JG57-OvJiszI4KUoBARQN4QAo6jVb4]and [https://mega.nz/file/IjM1WJ5Q#kGUobiLFeG6yyD7M2EYSnKLG4Ds1tiYNE1fpei8a7T4].

The comparison of results between the culture and the NGS of the 490 samples is in this published article

CRediT authorship contribution statement

Aishwariya Ramanathan: Writing – original draft. **Anusuya Marimuthu:** Investigation. **Lalanika M. Abayasekara:** Writing – review & editing. **Thurga Jeyaratnam:** Resources. **N. Vishvanath Chandrasekharan:** Project administration. **Jennifer Perera:** Writing – review & editing, Formal analysis. **Venkatasubramanian Ramasubramanian:** Validation. **Nandini Sethuraman:** Validation. **Sumudu Samarasinghe:** Software. **Varuna Peiris:** Software. **Vaz S. Gnanam:** Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Financial: This study was sponsored by Micro Genomics India Pvt Ltd for the validation of Bactfast and Fungifast products of

Credence Genomics Pvt Ltd. Author VSG is the Managing director of Micro Genomics India Pvt Ltd and Credence Genomics Pvt Ltd. Author AR is employee of Micro Genomics India Pvt Ltd. AM is past employee of Micro Genomics India Pvt Ltd. Author LMA is employee of Credence Genomics Pvt Ltd. SS, VP, TJ are past employee of Credence Genomics Pvt Ltd.Author VC is affiliated with Sri Lanka Institute of Biotechnology (SLIBTEC), Pitipana, Homagama, Sri Lanka.and past employee of Credence Genomics Pvt. Ltd. as a consultant in molecular biology at the time of the study. Author JP is a collaborator in the University of Colombo as the Professor of Microbiology, JP is also affiliated with Credence Genomics Pvt. Ltd. as a specialist in Medical Microbiology at the time of the study.

Acknowledgement

Apollo hospitals, Microbiology Laboratory for provision of the clinical material for the study and the culture results for the final comparison.

Apollo hospitals for the provision of blood samples.

Apollo research and Innovation for provision of ethical approvals and obtaining consent form for the blood sample collection from the patients.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34538.

References

[1] Y. Sakr, U. Jaschinski, X. Wittebole, T. Szakmany, J. Lipman, S.A. Ñamendys-Silva, I. Martin-Loeches, M. Leone, M.N. Lupu, J.L. Vincent, Investigators Icon, Sepsis in intensive care Unit patients: worldwide data from the intensive care over nations audit, Open Forum Infect. Dis. 5 (12) (2018 Nov 19) ofy313, https://doi.org/10.1093/ofid/ofy313. PMID: 30555852; PMCID: PMC6289022.

[2] A.M. Harris, A.M. Bramley, S. Jain, S.R. Arnold, K. Ampofo, W.H. Self, D.J. Williams, E.J. Anderson, C.G. Grijalva, J.A. McCullers, A.T. Pavia, R.G. Wunderink, K.M. Edwards, J.M. Winchell, L.A. Hicks, Influence of antibiotics on the detection of bacteria by culture-based and culture-independent diagnostic tests in patients hospitalized with community-acquired pneumonia, Open Forum Infect. Dis. 4 (1) (2017 Feb 10) ofx014, https://doi.org/10.1093/ofid/ofx014.

[3] Mobed Ahmad, Behzad Baradaran, Miguel de la Guardia, Mohammad Agazadeh, Mohammad Hasanzadeh, Mohammad Ahangarzadeh Rezaee, Jafar Mosafer, Ahad Mokhtarzadeh, Michael R. Hamblin, Advances in detection of fastidious bacteria: from microscopic observation to molecular biosensors, TrAC, Trends Anal. Chem. 113 (2019) 157–171.

[4] R.P. Dickson, J.R. Erb-Downward, H.C. Prescott, F.J. Martinez, J.L. Curtis, V.N. Lama, G.B. Huffnagle, Analysis of culture-dependent versus culture-independent techniques for identification of bacteria in clinically obtained bronchoalveolar lavage fluid, J. Clin. Microbiol. 52 (10) (2014 Oct) 3605–3613, https://doi.org/10.1128/JCM.01028-14. Epub 2014 Jul 30. PMID: 25078910; PMCID: PMC4187760.

[5] P.P. Bosshard, Incubation of fungal cultures: how long is long enough? Mycoses 54 (5) (2011 Sep) e539–e545, https://doi.org/10.1111/j.1439-0507.2010.01977.x. Epub 2011 May 23. PMID: 21605185.

[6] D.D. Rhoads, R.D. Wolcott, Y. Sun, S.E. Dowd, Comparison of culture and molecular identification of bacteria in chronic wounds, Int. J. Mol. Sci. 13 (3) (2012) 2535–2550, https://doi.org/10.3390/ijms13032535. Epub 2012 Feb 23. PMID: 22489109; PMCID: PMC3317672.

[7] P.D. Khot, D.L. Ko, D.N. Fredricks, Sequencing and analysis of fungal rRNA operons for development of broad-range fungal PCR assays, Appl. Environ. Microbiol. 75 (6) (2009 Mar) 1559–1565, https://doi.org/10.1128/AEM.02383-08. Epub 2009 Jan 9. PMID: 19139223; PMCID: PMC2655463.

[8] L. Barth Relle, others, Detection and identification of microorganisms by gene amplification and sequencing, Clin. Infect. Dis. 44 (8) (2007) 1108–1114, https://doi.org/10.1086/512818.

[9] S.N. Leaw, H.C. Chang, H.F. Sun, R. Barton, J.P. Bouchara, T.C. Chang, Identification of medically important yeast species by sequence analysis of the internal transcribed spacer regions, J. Clin. Microbiol. 44 (3) (2006 Mar) 693–699, https://doi.org/10.1128/JCM.44.3.693-699.2006. Erratum in: J Clin Microbiol. 2007 Mar;45(3):1079. PMID: 16517841; PMCID: PMC1393093.

[10] L.M. Abayasekara, J. Perera, V. Chandrasekharan, et al., Detection of bacterial pathogens from clinical specimens using conventional microbial culture and *16S* metagenomics: a comparative study, BMC Infect. Dis. 17 (2017) 631, https://doi.org/10.1186/s12879-017-2727-8.

[11] Z.G. Wei, X.D. Zhang, M. Cao, F. Liu, Y. Qian, S.W. Zhang, Comparison of methods for picking the operational taxonomic units from amplicon sequences, Front. Microbiol. 12 (2021 Mar 24) 644012, https://doi.org/10.3389/fmicb.2021.644012. PMID: 33841367; PMCID: PMC8024490;
[[11]b] Human Microbiome Project Consortium, Structure, function and diversity of the healthy human microbiome, Nature 486 (7402) (2012 Jun 13) 207–214, https://doi.org/10.1038/nature11234. PMID: 22699609; PMCID: PMC3564958.

[12] I. Jbara, M. Baysallar, A. Kiliç, S. Yetişer, B. Unay, C. Açikel, M. Yapar, L. Doğanci, Comparison of culture and polymerase chain reaction methods for the detection of Haemophilus influenzae, Streptococcus pneumoniae and Moraxella catarrhalis in cerebrospinal fluids and middle ear effusions], Mikrobiyol Bul 41 (4) (2007 Oct) 495–502. Turkish. PMID: 18173067.

[13] S. Schoonbroodt, J.L. Ichanté, S. Boffé, N. Devos, J.M. Devaster, L. Taddei, S. Rondini, A.K. Arora, T. Pascal, L. Malvaux, Real-time PCR has advantages over culture-based methods in identifying major airway bacterial pathogens in chronic obstructive pulmonary disease: results from three clinical studies in Europe and North America, Front. Microbiol. 13 (2023 Feb 24) 1098133, https://doi.org/10.3389/fmicb.2022.1098133. PMID: 36909845; PMCID: PMC10000296.

[14] O.K. Eser, S. Alp, A. Ergin, K. Ipçi, A. Alp, D. Gür, G. Hasçelik, Comparison of culture and real-time PCR methods in the detection of Streptococcus pneumoniae and Haemophilus influenzae in acute otitis media effusion specimens, Mikrobiyol Bul 46 (4) (2012 Oct) 676–681. Turkish. PMID: 23188581.

[15] * Asmita Kamble, Sawant, Shriya, Harinder Singh, 16S ribosomal rna gene-based metagenomics: a review, Biomedical Research Journal 7 (1) (January 2020) 5–11, https://doi.org/10.4103/BMRJ.BMRJ_4_20.

[16] C. Oliveira, E. Shakiba, D. North, M. McGraw, E. Ballard, M. Barrett-D'Amico, G. Glazko, Y. Rahmatallah, 16S rRNA gene-based metagenomic analysis of rhizosphere soil bacteria in Arkansas rice crop fields, Agronomy 12 (2022) 222, https://doi.org/10.3390/agronomy12010222.