A metagenomic analysis with oligotrophic enrichment approach for detecting specified microorganisms

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Abstract

In pharmaceutical manufacturing, significant benefit is conferred by the detection and identification of specific objectionable microorganisms (*i.e., Burkholderia cepacia* complex (BCC), *E. coli, Pseudomonas aeruginosa*, *Salmonella enterica, Staphylococcus aureus, Clostridia sporogenes, Candida albicans, Mycoplasma*), which were previously missed or not identified by culture-dependent methods. We developed a metagenomic analysis coupled with culture enrichment in order to (i) detect "specific microorganisms"; (ii) evaluate Tryptic Soy Broth (TSB) and 1/10× TSB for the recovery of specific microorganisms; (iii) assess whether co-inoculation of BCC in water can enhance recovery of specified microorganisms; and (iv) identify metabolic activities of BCC in water. In total, 589-996 genera were identified in 12 water samples taken from a cold water fountain with *Bacillus* sp. (97%) in TSB and *Stenotrophomonas* spp. (97%) in 1/10 TSB, which accounted for primarily recovered genera after a 72-hour pre-enrichment at 23 °C. Likewise, we also detected lower abundance of specific organisms, *Clostridium* spp., *Burkholderia* spp., and *Staphylococcus* spp. (0.04 - 0.07%) in TSB and *Burkholderia* spp., *Pseudomonas* spp., *Salmonella* spp., *Staphylococcus* spp. and *Escherichia* spp. (0.01 - 1.73%) in 1/10× TSB. Co-inoculation with BCC yielded a higher recovery rate of *Pseudomonas* spp. compared to uninoculated controls in 1/10× TSB. The abundance of BCC in water was found to be related to the toluene degradation (PWY-5180 and PWY-5182) pathways. These initial results demonstrate that the quasimetagenomic approach can be a powerful tool for assessing and monitoring specific organisms, such as BCC in non-sterile pharmaceutical products.

Introduction

- Non-sterile water-based drug and non-drug products have been shown to be contaminated with objectionable pathogens and have caused product recalls within the U.S. A report published in 2019 surveying FDA recalls from 2012 to 2019, *Burkholderia* spp. were the number one reason for non-sterile drug recalls (105 recalls) followed by *Ralstonia pickettii* (45 recalls) and *Salmonella* spp. (28 recalls). Unidentified microbial contamination accounted for 77% of non-sterile and 87% of sterile drug recalls, indicating extremely poor microbiology practices. Overall, these pioneering surveillance reports clearly showed that a significant proportion of microbial contaminants were left unidentified. The presence of certain microorganisms in non-sterile preparations may not only have the potential to reduce or even inactivate the therapeutic activity of drug products but also consequently adversely affect patients health.
- U.S. Pharmacopeia (USP) <1111> sets acceptance criteria for the presence of certain microorganisms in non-sterile preparations based on the route of administration {USP, 2016 #38}. Furthermore, USP <61> and <62> testing is designed to demonstrate compliance with these requirements by quantifying the presence of specified microorganisms (*i.e., E. coli, Pseudomonas aeruginosa*, *Salmonella* spp., *Staphylococcus aureus, Clostridia* spp., *Candida albicans* and the *Enterobacteriaceae* family), *Burkholderia cepacia* complex (USP <60>) and *Mycoplasma* (USP <63>). Although science has conclusively shown that culture-based detection misses an abundance of microorganisms , Current Good Manufacturing Practice (cGMP) expects that investigations cannot progress unless a colony is recovered, and therefore only culture-based methods (*e.g.*, for determining sterility and microbial limits) are considered GMP. Additionally, USP has traditionally relied on the least common denominator for test methods.
- Our study mainly aims to describe specified microorganisms and BCC in enriched water samples. The objectives of this study were (i) to detect "specified microorganisms" (*i.e.,* BCC (USP <60>), *E. coli, Pseudomonas aeruginosa*, *Salmonella enterica, Staphylococcus aureus, Clostridium sporogenes, Candida albicans* (USP <62>), *Mycoplasma* (USP <63>)); (ii) to evaluate Tryptic Soy Broth (TSB) and 1/10× TSB for the recovery of specified microorganisms; (iii) to understand the synergistic effects of BCC spiked for recovery of specified microorganisms; and (iv) to identify BCC metabolic pathway in BCC-spiked water.

Methodology

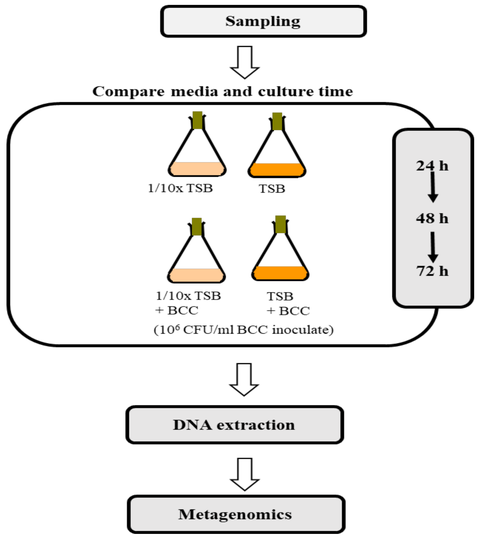

Fig 1. Overview of the metagenomics study

Results

Table 1. Cold fountain water samples included in the study.

Sample name	Medium	BCC added	Culture time	DNA conc. (ng/µl)	Final DNA conc. (ng/µl)	Average Library size (bp)	Average GC content (%)	DNA paired end reads; Raw	Final sequencing libraries
TSB-24 h	TSB		24 h	169.2	23.40	657	35	26,421,188	9,240,862
TSB + BCC -24 h	TSB	BCC	24 h	412.0	16.10	742	66	28,641,055	23,660,634
1/10× TSB-24 h	1/10× TSB		24 h	Too low; 404.0*	23.40	744	49	21,103,554	9,529,702
1/10× TSB + BCC-24 h	1/10× TSB	BCC	24 h	432.0	19.30	648	66	34,121,260	28,925,622
TSB-48 h	TSB		48 h	212.0	21.20	703	34	24,216,049	8,481,220
TSB + BCC-48 h	TSB	BCC	48 h	928.0	22.20	713	66	25,418,445	22,075,138
1/10× TSB-48 h	1/10× TSB		48 h	940.0	19.70	697	66	29,205,073	22,207,514
1/10× TSB + BCC-48 h	1/10× TSB	BCC	48 h	624.0	18.60	636	66	40,029,112	34,094,584
TSB-72 h	TSB		72 h	230.0	20.40	713	34	18,508,113	7,052,140
TSB + BCC-72 h	TSB	BCC	72 h	1220.0	19.20	740	66	24,901,170	21,671,352
1/10× TSB-72 h	1/10× TSB		72 h	696.0	12.10	765	66	34,465,670	24,475,759
1/10× TSB + BCC-72 h	1/10× TSB	BCC	72 h	692.0	20.00	699	66	26,924,498	23,133,632

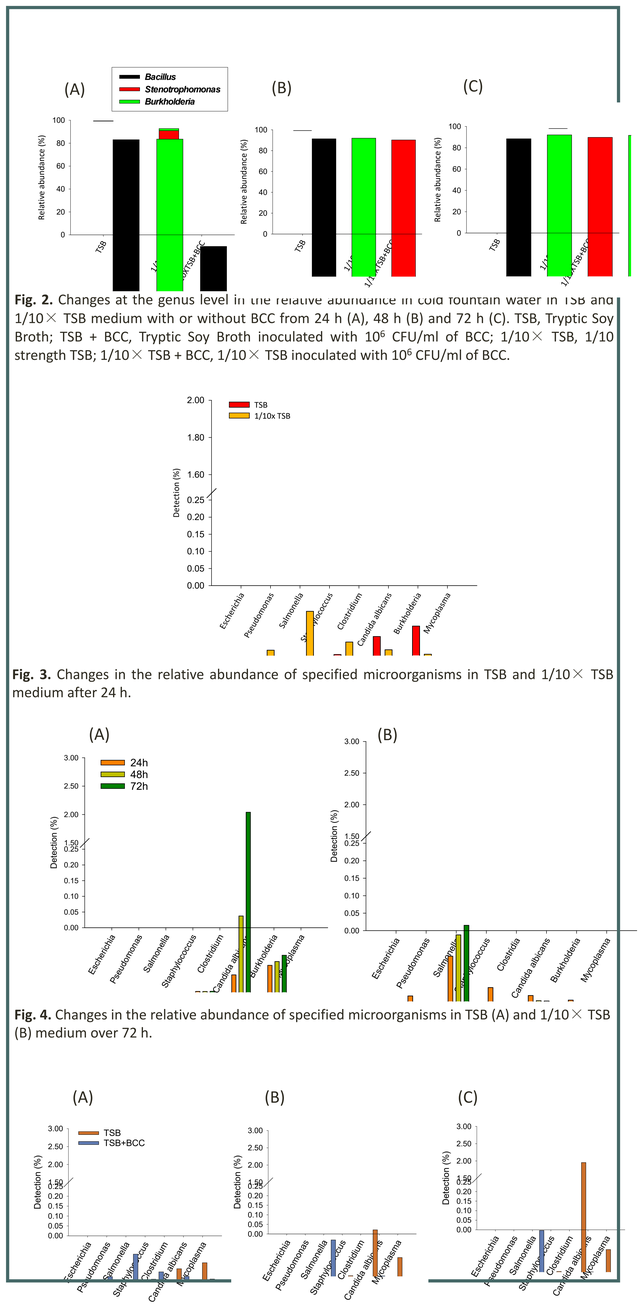

Fig. 2. Changes at the genus level in the relative abundance in cold fountain water in TSB and 1/10× TSB medium with or without BCC from 24 h (A), 48 h (B) and 72 h (C). TSB, Tryptic Soy Broth; TSB + BCC, Tryptic Soy Broth inoculated with 10^6 CFU/ml of BCC; 1/10× TSB, 1/10 strength TSB; 1/10× TSB + BCC, 1/10× TSB inoculated with 10^6 CFU/ml of BCC.

Fig. 3. Changes in the relative abundance of specified microorganisms in TSB and 1/10× TSB medium after 24 h.

Fig. 4. Changes in the relative abundance of specified microorganisms in TSB (A) and 1/10× TSB (B) medium over 72 h.

Fig. 5. Changes in the relative abundance of specified microorganisms in TSB added 10^6 CFU/ml BCC (TSB + BCC) at 24 h (A), 48 h (B) and 72 h (C).

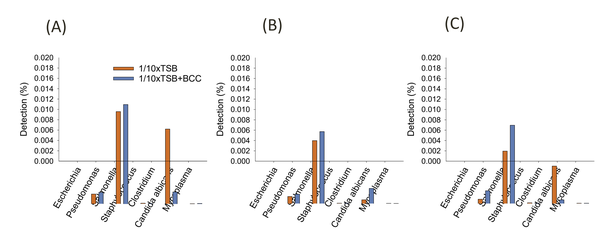

Fig. 6. Changes in the relative abundance of specified microorganisms in 1/10× TSB and 1/10× TSB + BCC at 24 h (A), 48 h (B) and 72 h (C).

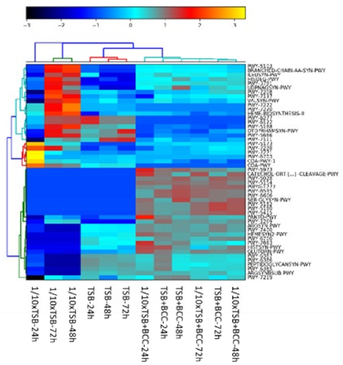

Fig. 7. Heatmap of relative abundance of the 50 most abundant metabolic pathways in the metagenomes found in water collected from a cold water fountain.

Conclusion

- Specified microorganisms (*i.e.,* BCC, *E. coli, Pseudomonas aeruginosa*, *Salmonella enterica, Staphylococcus aureus, Clostridium sporogenes, Candida albicans,* and *Mycoplasma*) were observed less than 1.8% of relative sequencing read abundance in TSB and 1/10× TSB after 24 h. It looks like it's more than 1.8% in 1/10× TSB.
- In TSB, *Clostridium* spp., *Burkholderia* spp., and *Staphylococcus* spp. were observed 0.07%, 0.06%, and 0.04%, respectively. In 1/10× TSB, *Burkholderia* spp. (1.73%) was predominant in the samples. *Pseudomonas* spp., *Salmonella* spp., *Staphylococcus* spp. and *Escherichia* spp. were observed 0.09%, 0.03%, 0.01% and 0.01%, respectively.
- In TSB + BCC, *Pseudomonas* spp. were the most dominant and observed 0.09% at 24 h , in 1/10× TSB + BCC, *Pseudomonas* spp., *Staphylococcus* spp., and *Escherichia* spp., were also observed 0.014, 0.01, and 0.001%, respectively.
- Metagenomic analysis of BCC-spiked samples has provided new insights into metabolic pathways in water samples vs. BCC-spiked samples.

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