Journal club presentation

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RESEARCH ARTICLE

Identification of Gram negative non-fermentative bacteria: How hard can it be?

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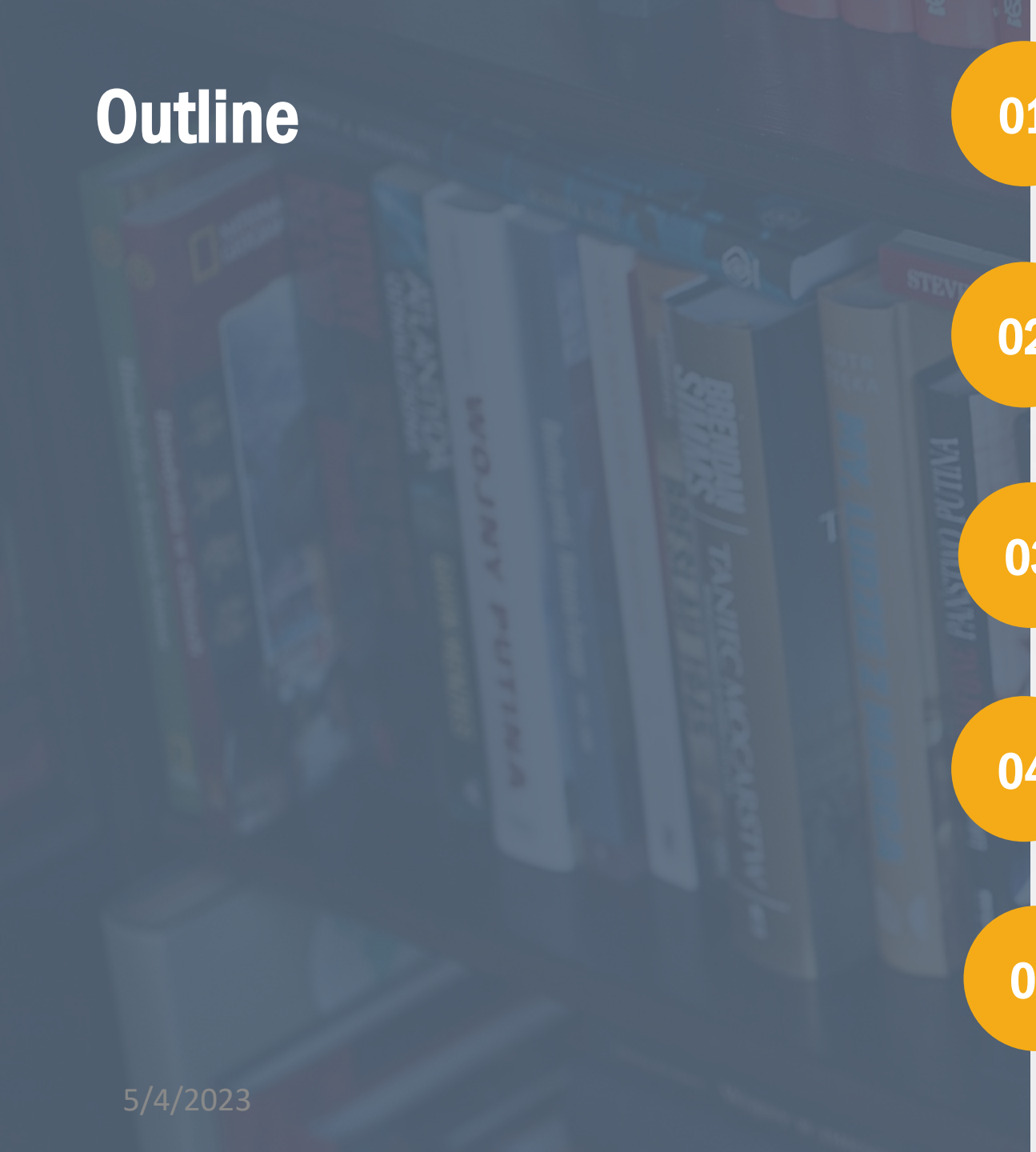
1	PLoS Medicine ISSN/ISBN: 1549-1277, 1549-1676	5%		ProQuest	11.613	Q1	15.00	Q1	242	ISI, Scopus, PubMed, Embase, DOAJ	
2	PLoS Biology ISSN/ISBN: 1544-9173, 1545-7885	5%	Genetics Biochemistry + 4 more ...	ProQuest	9.593	Q1	14.90	Q1	281	ISI, Scopus, PubMed, Embase, DOAJ	
3	PLoS Pathogens ISSN/ISBN: 1553-7366, 1553-7374		Parasitology Virology + 4 more ...	ProQuest	7.464	Q1	10.50	Q1	219	ISI, Scopus, PubMed, Embase, DOAJ	
4	PLoS Genetics ISSN/ISBN: 1553-7390, 1553-7404		Genetics Biology	ProQuest	6.020	Q1	9.30	Q1	244	ISI, Scopus, PubMed, Embase, DOAJ	
5	PLoS Neglected Tropical Diseases ISSN/ISBN: 1935-2727, 1935-2735		Occupational M... Environmental ... + 2 more ...	ProQuest	4.781	Q1	6.80	Q1	143	ISI, Scopus, PubMed, Embase, DOAJ	
6	PLoS Computational Biology ISSN/ISBN: 1553-734X, 1553-7358		Genetics Biology + 1 more ...	ProQuest	4.779	Q1	6.60	Q1	191	ISI, Scopus, PubMed, Embase, DOAJ	
	PLoS ONE		General Medicine							ISI, Scopus,	

Outline



01 Introduction

02 Methods

03 Results

04 Discussion & Conclusions

05 Critique

Title

RESEARCH ARTICLE

Identification of Gram negative non-fermentative bacteria: How hard can it be?

Introduction

- The prevalence of **bacteremia** caused by Gram negative non-fermentative (GNNF) bacteria has been **increasing globally** over the past decade.

- Many studies have investigated their **epidemiology** but focus on the **common GNNF** including *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

- **Knowledge** of the **uncommon GNNF bacteremias** is very limited.

Introduction

- The GNNF are widely **distributed in the environment** and have become increasingly common isolates in the **clinical laboratory.**

- Medically, their **pathogenic potential** has been **proved** beyond doubt by their frequent isolation from **clinical material** and their **association with disease**.

- They are not only isolated in **nosocomial settings**, mainly **affecting immuno-comprised hosts**, but also are **opportunistic infections** causing **invasive disease** in rural communities.

Introduction

- This group of bacteria are **difficult to treat** because of **widespread antibiotic resistance**.

- These bacteria are **taxonomically heterogeneous**, and many isolates **are** not satisfactorily **identified** by the **standard biochemical assays** and conventional microbiology because of **overlapping phenotypic characteristics**.

- Thus, their **accurate identification** **to the species level** is important for appropriate **patient management.**

Introduction

Automated systems that perform organism identification and antimicrobial susceptibility testing are now the mainstay of clinical microbiology laboratories. The recent implementation of the BD Phoenix and a MiSeq next generation sequencing (NGS) instrument in our laboratory-based surveillance activities has allowed us to re-examine a subset of previously unidentified Gram negative bacilli and determine which genera are contributing to bloodstream infections in rural, agrarian populations in Thailand. An understanding of the uncommon Gram negative non-fermentative (GNNF) bacteria causing invasive disease in these communities should assist in diagnosis and treatment and possibly impact patient outcomes.

Methods

01

Isolate collection

02

BD Phoenix system procedures

03

Whole genome sequencing

Isolate collection

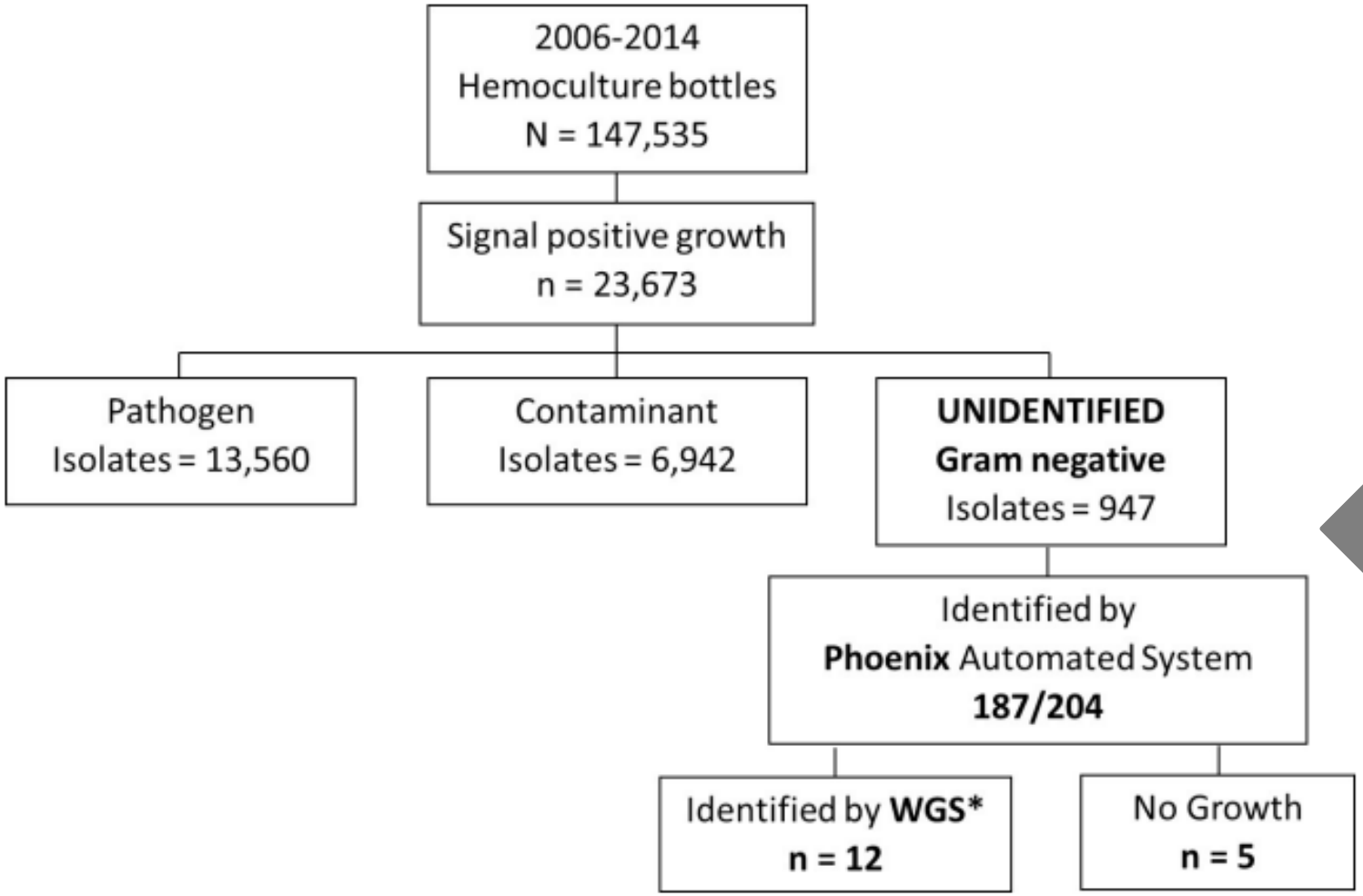


Fig 1. Flow diagram denoting blood culture results from blood-stream infection surveillance in two rural Thai provinces from 2006–2014. *WGS–whole genome sequencing.

https://doi.org/10.1371/journal.pntd.0007729.g001

A random subset of approximately 20% (n = 204) of the 947 unidentified organisms were used in this study.

BD Phoenix system procedures

BD Phoenix system procedures

Upon removal from the freezer, isolates were grown on tryptic soy agar with 5% sheep blood. The inoculum for the Phoenix Gram-negative identification and susceptibility combination panel NMIC/ID 55 was prepared according to the manufacturer's instructions and panels were sealed, logged, and loaded into the instrument for incubation at 35˚C (http://legacy.bd.com/ds/technicalCenter/clsi/clsi-Phoenix_GramNegative_V5.15_V4.31.pdf). Kinetic, colorimetric, and fluorescent signals were automatically collected by the instrument every 20 minutes until results were completed. The Phoenix system leads to an identification result when a species or group of species is identified with a ≥90% confidence level [3]. Antimicrobial susceptibility testing (AST) breakpoints were interpreted using the Clinical and Laboratory Standards Institute (CLSI) guidelines from 2016 [4].

Whole genome sequencing

DNA was extracted from bacterial colonies using the manual Qiagen DNA Mini Kit. Libraries were prepared employing the Illumina Nextera XT DNA Library Prep Kit according to the manufacturer's instructions. Whole genome sequencing was performed on the Illumina MiSeq sequencer (Illumina, San Diego, CA) using Illumina MiSeq Reagent Kit version 2.0 and 300 cycles paired-end runs.

Sequences have been deposited in the NCBI Sequence Read Archive and can be accessed through Biosample identifiers SAMN11784141–52.

sequencing (WGS) on a MiSeq platform and data analysis performed using 3 web-based platforms: Taxonomer, CGE KmerFinder and One Codex.


RESULTS

Results

Approximately 7% (947/14,507) of all clinical isolates could not be identified by standard biochemical methods. A proportion of these, denoted as "unknown" (204/947 (21.5%); Fig 1), were randomly selected and examined using the BD Phoenix automated identification and susceptibility testing system. Identification to at least the genus level was achieved for 187/204 (91.7%) (Fig 2; S1 Tables). The largest number of isolates identified as *Achromobacter* species (46/204; 22.5%), with nearly 70% of cases being in adults ≥50 years of age (32/46, Table 1). One case appeared to be hospital onset (defined as positive blood cultures obtained >2 days after hospital admission), with blood obtained for blood culture 5 days after admission. All others were community-acquired infections. AST data was available for 36 isolates (78.3%) and all showed resistance to ampicillin, amoxicillin-clavulanate, aztreonam, and cefazolin. Cefepime resistance was determined in 14/36 isolates with the remaining 18 isolates scoring as intermediate (Fig 3; S1 Tables). Ten *Achromobacter* isolates identified by Phoenix had no associated AST data as isolate growth was too slow, preventing the control from reaching the required cutoff value and terminating that portion of the panel. On re-test a similar result was obtained.

Pseudomonas species were identified in 35/204 isolates (17.2%) of which the most common were *P. putida* (11; 31.4%), and *P. pseudoalcaligenes* 8 (22.9%). *P. oryzihabitans* and *P. aeruginosa* had 5 and 4 isolates respectively, and one isolation each of *P. luteola*, *P. mendocina* and *P. stutzeri*. Three *Pseudomonas* isolates were not speciated. The majority of isolates were not able to meet the growth criteria required in the AST control, even upon re-testing, and susceptibility profiles were only available for 9/35 (25.7%) isolates. These were 100% resistant to ampicillin, amoxicillin/clavulanate (augmentin), cefazolin, and cefoxitin, as expected with intrinsic resistance to these antibiotics. All 9 were sensitive to amikacin, cefepime, imipenem and piperacillin/tazobactam (Fig 3; S1 Tables). Twenty percent of cases (7/35) were in children <5 years old (Table 1); and 3/4 likely hospital onset were in children <5 years old.

The third most common isolates were *Moraxella* species (14/204 cases (6.9%)), with 5 being hospital onset infections, and most cases occurring in adults ≥50 years old (9/14; 64.3%). AST profiles were not available as *Moraxella* species are not included in the Phoenix AST database [8].

Other genera identified are *Sphingomonas paucimobilis* (13/204 isolates), twelve isolations of *Pasteurella* species (7 *P. multocida*, 2 *P. aerogenes*, and 3 *P. pneumotropica*), and 9 isolations of *Ochrobactrum anthropi*.

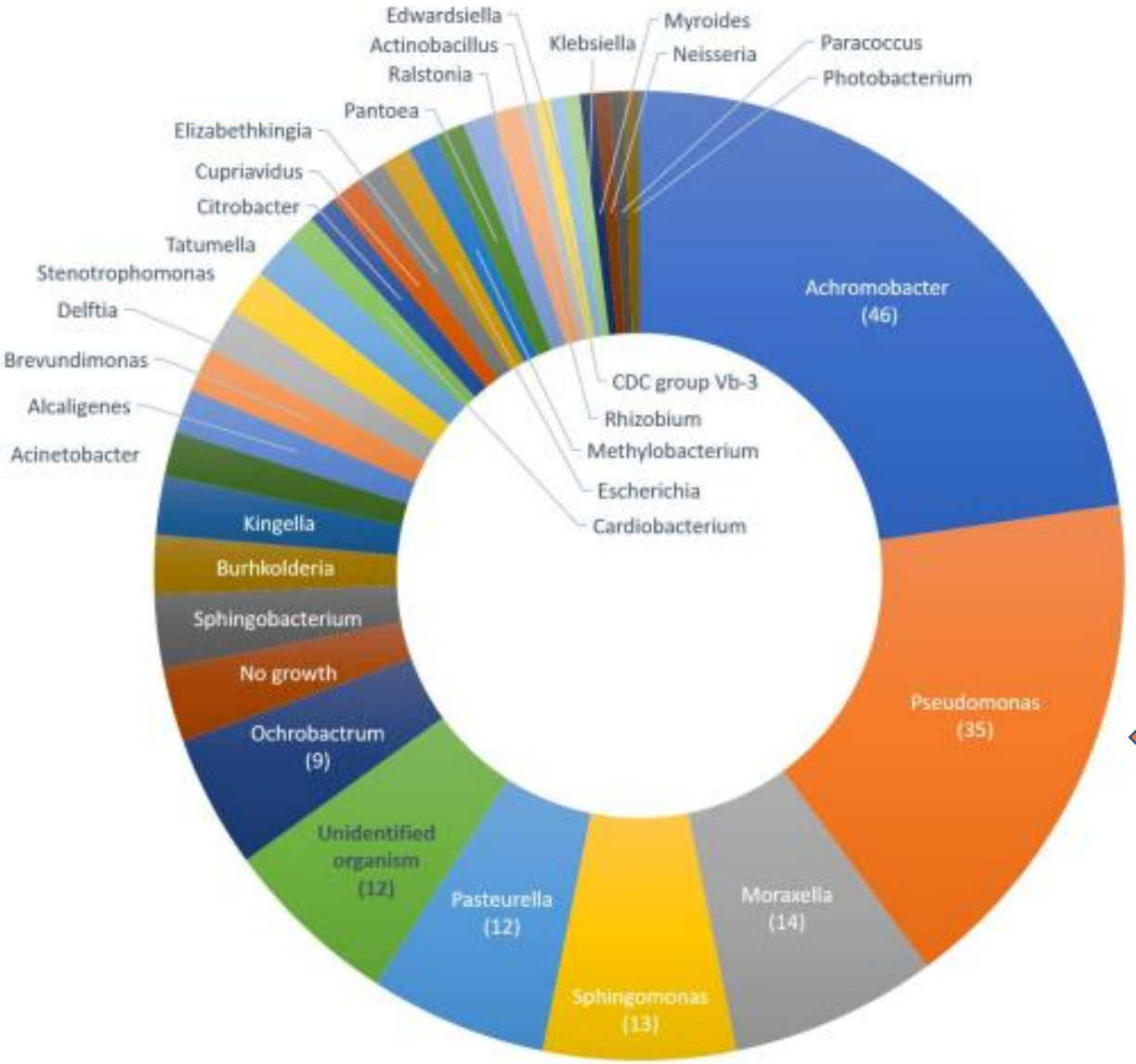


Fig 2. Distribution of previously unidentified isolates at the genus level using BD Phoenix automated identification system.

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P. putida (11; 31.4%)
P. pseudoalcaligenes 8 (22.9%).
P. oryzihabitans
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and one isolation each of
P. luteola,
P. mendocina
P. stutzeri.
Three Pseudomonas isolates were not speciated.

Table 1. Demographic data associated with the 204 unidentified isolates processed using the BD Phoenix automated identification system, focusing on commonly identified genera.

	GNNF Phoenix ID (n = 204)		*Achromobacter* spp. (n = 46)		*Pseudomonas* spp. (n = 35)		*Moraxella* spp. (n = 14)	
	n	%	n	%	n	%	n	%
Age Group **n**	**188**	**92.2**	**44**	**95.7**	**33**	**94.3**	**13**	**92.9**
(years) 0–4	20	10.6	0	0	7	21.2	1	7.7
5–19	6	3.2	1	2.3	2	6.1	0	0
20–49	42	22.3	11	25.0	4	12.1	3	23.1
50–74	89	47.3	23	52.3	18	54.5	4	30.8
75+	31	16.5	9	20.4	2	6.1	5	38.5
Sex **n**	**73**	**35.8**	**19**	**41.3**	**11**	**31.4**	**4**	**28.6**
Male	37	50.7	8	42.1	6	54.5	3	75.0
Female	36	49.3	11	57.9	5	45.5	1	25.0
Onset[a] **n**	**188**	**92.2**	**41**	**89.1**	**32**	**91.4**	**13**	**92.9**
Community Onset	110	58.5	40	97.6	28	87.5	8	61.5
Hospital Onset	78	41.5	1	2.4	4	12.5	5	38.5
Outcome[b] **n**	**126**	**61.8**	**37**	**84.1**	**22**	**62.6**	**9**	**64.3**
Complete recovery	14	11.1	3	8.1	3	13.6		
Improved	76	60.3	22	59.5	12	54.5	7	77.8
Not improved	23	18.3	7	18.9	6	27.3	1	11.1
Death	13	10.3	5	13.5	1	4.5	1	11.1
Site **n**	**204**	**100**	**46**	**100**	**35**	**100**	**14**	**100**
Nakhon Phanom	140	68.6	41	89.1	20	57.1	11	78.6
Sa Kaeo	64	31.4	5	10.9	15	42.9	3	21.4

Table 2. Summary of patient antibiotic use prior to admission and time-to-positivity (TTP) for blood culture.

		GNNF Phoenix ID (n = 204)		*Achromobacter* spp. (n = 46)		*Pseudomonas* spp. (n = 35)		*Moraxella* spp. (n = 14)	
		n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Antibiotic use within 72h admission (Self-report)	**n**	**183**	**89.7**	**44**	**95.6**	**31**	**88.6**	**12**	**85.7**
	Yes	133	71.1	26	59.1	23	74.2	10	83.3
	No	31	16.9	12	27.3	7	22.6	2	16.7
	Not sure	19	10.3	6	13.6	1	3.2	0	0
Blood culture TTP*	**n**	**176**	**86.3**	**44**	**95.6**	**31**	**86.1**	**12**	**65.7**
(hours)	≤ 24	25	14.2	1	2.3	4	12.9	3	25.0
	25–48	86	48.9	22	50.0	17	54.8	4	33.3
	> 48	65	36.9	21	47.7	11	35.5	5	41.7

* TTP defined as the time from the start of blood culture bottle incubation to a positive signal.

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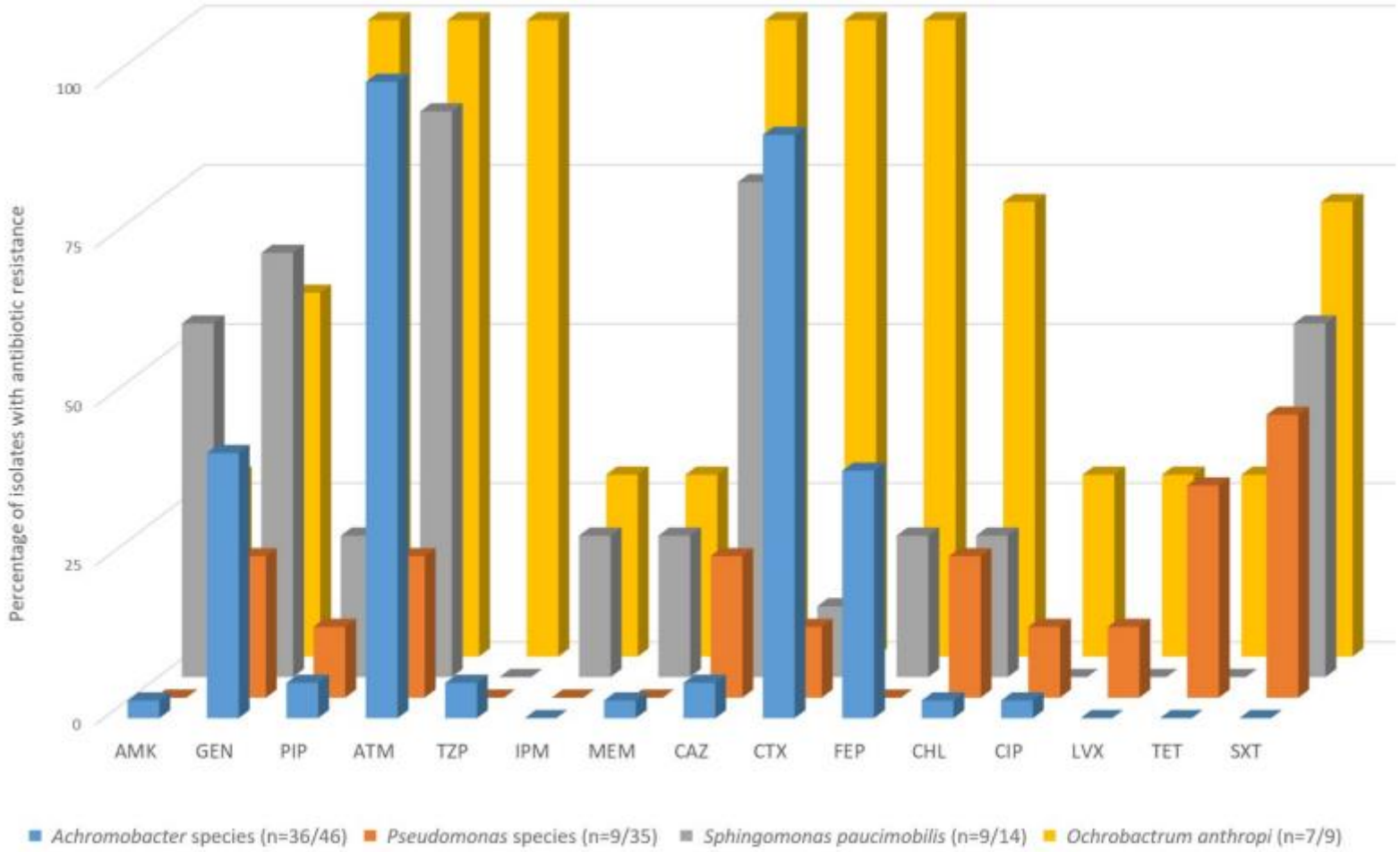

Fig 3. Antibiotic resistance levels by organism using the BD Phoenix identification system.

- **Moraxella** (n = 14) and **Pasteurella** (n = 12) species are not included in the **BD Phoenix AST database** and therefore this data is not available.

Table 3. Summary of isolate identification using 3 different web-based platforms on whole genome sequencing short read raw data files.

ID	CGE: KmerFinder https://cge.cbs.dtu.dk/services/KmerFinder/	Taxonomer https://www.taxonomer.com/	One Codex https://onecodex.com/
NA45072	*Acinetobacter nosocomialis*	*Wohlfahrtiimonas chitiniclastica*	*Wohlfahrtiimonas chitiniclastica*[1]
NA45230	Genus: *Acinetobacter*	Genus: *Acinetobacter*	Genus: *Acinetobacter*
NA45737	*Moraxella osloensis*	Genus: *Enhydrobacter*	Genus: *Enhydrobacter*
NA48754	*Delftia acidovorans*	Genus: *Delftia*	Genus: *Delftia*
NA55273	*Burkholderia cenocepacia*	Genus: *Burkholderia*	*Burkholderia cenocepacia*[1]
NA62451	*Roseomonas gilardii*	Family: Acetobacteraceae	*Roseomonas gilardii*[1]
NA62784	*Roseomonas gilardii*	Family: Acetobacteraceae	*Roseomonas gilardii*
NA64181	*Pseudomonas oryzihabitans*	Genus: *Pseudomonas*	Genus: *Pseudomonas*
NA66303	***Laribacter hongkongensis***	***Laribacter hongkongensis***	***Laribacter hongkongensis***[1]
NA69389	Genus: *Chryseobacterium*[*]	Genus: *Chryseobacterium*	*Flavobacteriales bacterium*
SA27898	*Moraxella osloensis*[*]	*Enhydrobacter aerosaccus*	*Moraxella atlantae*[1]
SC13199	*Acinetobacter indicus*	Genus: *Acinetobacter*	*Acinetobacter indicus*

[*]Low confidence identification: only 1–5% of reads map to the designated organism.

[1]One Codex high confidence identification. The sample contains >50% unique genomic content of that organism

https://doi.org/10.1371/journal.pntd.0007729.t003

A total of 17/204 (5.9%) isolates could not be identified by the Phoenix system, five because of no growth. **Characterization of the remaining 12 isolates was undertaken through WGS on an Illumina MiSeq platform.**

Discussion

Discussion

The correct and rapid identification of bacteria in a clinical microbiology laboratory, along with antimicrobial sensitivity testing, is an essential step towards the correct treatment of patients. Our analysis of 9 years of blood culture surveillance data shows that traditional diagnostic methodologies resulted in a positive identification in 93.5% of isolates (13,506/14,507). A fair proportion of the unidentified isolates were GNNF bacteria which often present as colorless/pale colonies that lack key metabolic characteristics, which impairs their identification by conventional methods. In 2015, the BD Phoenix automated identification and susceptibility testing system was installed in our laboratory and we retrospectively ran 204 previously unidentified GNNF isolates through the system. This allowed us to identify a further 91.7% (187/204) of isolates to at least the genus level. Due to their taxonomic complexity and high phenotypic similarity, identification to the species level represents a challenge even for the automated systems. In the majority of cases isolates were ubiquitous environmental organisms such as *Achromobacter* and *Sphingomonas* that are likely opportunistic pathogens [9–11].

There are an increasing number of case reports and reviews published suggesting a global increase in achromobacterial disease [10, 12], even so most clinicians remain unclear to their significance (and that of other environmental organisms) when clinically isolated. Additionally, effective treatment can be challenging due to these organism's intrinsic and acquired multidrug resistance patterns. Our surveillance populations were from rural, agrarian provinces so infections with environmental organisms is not surprising in people who are occupationally exposed. Organisms from over 30 genera were identified. *Moraxella* (14/204) and *Pasteurella*

this study. The biggest challenge we faced with the introduction of NGS to our laboratory was the data analyses. Laboratory scientists generally lack experience in NGS short-read sequence bioinformatics and in low- or middle- income countries, laboratory computing resources are often limited and unable to handle large data sets. Fortunately, there are rapid, user-friendly web-based tools that can be applied without large investments in trained personnel or computational infrastructure. We chose three services accessible through personal computers with no requirements for computational infrastructure on the user side. For both the Phoenix automated identification system and the NGS web-based tools, the bacterial identification is highly dependent on the reference databases used. As long as there are similar microbes in the database unknowns can be identified. We believe the differences in performance between the

of strains. Kirstahler et al. [23] found that 43% of bacterial reference genomes, particularly incomplete ones, contained ambiguous sequences and removing these from databases reduced the number of false positive hits. This supports the need for curated microbial genome databases. Our results illustrate that reads derived from taxa that are absent from databases can result in false-negative and false-positive classifications, especially at the genus and species level. Different measures are provided in the outputs from the three programs and no parame-

The use of WGS in public health or clinical microbiology laboratories would provide unparalleled improvements in pathogen identification, antibiotic resistance detection, and outbreak investigations, however, this capacity is severely hindered by lack of common standards. Regulatory agencies have not yet provided (or even proposed) standard guidelines, testing has not been standardized, and benchmarks have not been set. External quality assurance and proficiency testing programs are in progress. Analytical pipelines with well-curated, continually updated reference database are also important components to implementation. Once these hurdles have been achieved the incorporation of NGS into clinical and public health routine workflows is achievable.

Conclusion

The GNNF bacteria are of increasing concern in the clinical setting, and our inability to identify these organisms and determine their AST profiles will impede treatment. Databases for automated identification systems and sequencing annotation need to be improved so that opportunistic organisms are better covered.

Positive points of the article

01 Complete and comprehensive statement of the introduction and purpose of the study.

02 A complete and comprehensive description of sample collection details and method.

03 Using a large sample size.

04 Design great tables and graphs to show results.

05 Having a strong and comprehensive discussion section.

Weaknesses of the article

01 Not using other articles to compare this study with other researches that have been done in this field.

02 Not stating limitations.

03 Restating the results in the discussion section.

04 Not having a separate discussion and conclusion section.

05 Not offering perspective for future studies in this field.


Thank You
For Your Atten