RESEARCH NOTE

REVISED Air sampling to assess potential generation of aerosolized viable bacteria during flow cytometric analysis of unfixed bacterial suspensions [version 2; peer review: 1 approved, 2 approved with reservations]

Previously titled: Air sampling during flow cytometric analysis of unfixed bacterial suspensions; a risk assessment

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Abstract

This study investigated aerosolized viable bacteria in a university research laboratory during operation of an acoustic-assisted flow cytometer for antimicrobial susceptibility testing by sampling room air before, during and after flow cytometer use. The aim was to assess the risk associated with use of an acoustic-assisted flow cytometer analyzing unfixed bacterial suspensions. Air sampling in a nearby clinical laboratory was conducted during the same period to provide context for the existing background of microorganisms that would be detected in the air. The three species of bacteria undergoing analysis by flow cytometer in the research laboratory were *Klebsiella pneumoniae, Burkholderia thailandensis* and *Streptococcus pneumoniae*. None of these was detected from multiple 1000 L air samples acquired in the research laboratory environment. The main cultured bacteria in both locations were skin commensal and environmental bacteria, presumed to have been disturbed or dispersed in laboratory air by personnel movements during routine laboratory activities. The concentrations of bacteria detected in research laboratory air samples were reduced after interventional cleaning measures were introduced and were lower than those in the diagnostic clinical microbiology laboratory. We conclude that our flow cytometric analyses of unfixed suspensions of *K. pneumoniae, B. thailandensis* and *S. pneumoniae* do not pose a risk to cytometer operators or other personnel in the laboratory but caution against extrapolation of our results to other bacteria and/or different flow cytometric experimental procedures.

Keywords

biosafety, laboratory biocontainment, bacteria, risk assessment, flow cytometer

Open Peer Review

Reviewer Status ✔ ? ?

	Invited Reviewers		
	1	**2**	**3**
REVISED **version 2** published 27 Feb 2018	✔ report	? report	? report
version 1 published 06 Nov 2017	? report	? report	✘ report

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Competing interests: Dr CF Carson is employed by the University of Western Australia and Dr TJJ Inglis is employed by the University of Western Australia and the state pathology service provider, PathWest Laboratory Medicine WA.

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REVISED **Amendments from Version 1**

This latest version of the manuscript addresses points raised by the reviewers, including the need to reflect the intent of this study more clearly in the title, abstract and body text.

We have clarified the rationale for comparison of detectable bacteria during use of the acoustic-assisted flow cytometer in our research laboratory, and levels of detectable airborne bacteria in the nearby clinical laboratory.

The clinical laboratory provides an important context for this work as it is the intended destination of a future flow-cytometer assisted antimicrobial susceptibility test capability.

Supplementary Figure 1 has been added to this version 2. It provides a floor plan of the two locations where air sampling was conducted in this study. The figure clarifies the spatial relationships mentioned in the text and addresses points raised by the referees.

See referee reports

Introduction

Flow cytometry techniques have been used to analyze bacteria for several decades[1,2], and for assessing the effects of antimicrobial agents since the 1980s[3–5]. The bacteria analyzed then included species that can be safely handled on an open bench in a suitably equipped microbiology laboratory, providing a series of standard biosafety procedures are adhered to[6]. Concerns about laboratory biosafety and containment increased after 2001[7,8] and led to higher physical containment levels for select biothreat agents and some bacterial species such as *Neisseria meningitidis,* prone to transmission by aerosols generated during laboratory procedures[9,10]. Given these concerns about bioaerosol transmission risks, it is not surprising that standards for bioaerosol risk assessment and mitigation have been recommended for fluorescence-activated cell sorting protocols[11,12]. Our use of flow cytometry was not for cell sorting, but for a less aerosol-prone cellular analysis. We commenced use of our analytic flow cytometer in a physical containment level two laboratory while developing a flow cytometry-assisted antimicrobial susceptibility test (FAST) assay method with *Klebsiella pneumoniae*[13]. Though the cytometer we used had no cell sorting function and therefore would not generally produce aerosols,[14,15] we decided to conduct an assessment to confirm that viable bacteria are not aerosolized during use before progressing with any analysis of potentially more hazardous aerosol-transmitted species such as *Neisseria meningitidis, Mycobacterium tuberculosis* and *Burkholderia pseudomallei*.

This study aimed to detect aerosolized viable bacteria during operation of an acoustic-assisted flow cytometer and to compare the detected bacteria with those cultured from air samples collected in the same laboratory space when the cytometer was not in use, and in a diagnostic clinical microbiology laboratory.

Methods

Laboratory locations. Two laboratory locations in adjacent buildings were used. One was a university research laboratory approximately 54.7 m^2 and 145.5 m^3 equipped with two acoustic-assisted flow cytometers, and two class two biosafety cabinets, peripheral benches and one central bench. One of the flow cytometers is housed and used in a biosafety cabinet while the other one, the focus of this study, is on the open bench. The other location was a large, open plan clinical laboratory microbiology laboratory approximately 400.5 m^2 and 1081.4 m^3 operating a range of high throughput bacteriology procedures serving an on-campus 700 bed teaching hospital and an extensive regional hospital network. Notably, the clinical laboratory does not have an acoustic-assisted flow cytometer. Both laboratories were air-conditioned and equipped with high efficiency particulate air filtration on external air outlets. Detailed information about the number of complete air exchanges per hour was not available for either location.

The clinical laboratory was included for comparative purposes. While the study aimed to determine if aerosolized viable target bacteria (*Burkholderia thailandensis*, *K. pneumoniae* and *Streptococcus pneumoniae*) could be detected in air in the research laboratory, the likelihood was that other microorganisms, commonly present in indoor air, would be detected. Published data regarding the range and concentration of bacteria present in air in microbiology laboratories, either research or clinical, are scarce. Without data for comparison, data on the viable bacteria detected in air samples from the research laboratory would be entirely without context. Work practices in the clinical laboratory are designed to maintain a safe working environment and the background level of viable bacteria detected in clinical laboratory air could therefore serve as a proxy indicator of the acceptable level of viable bacteria in air in the research laboratory.

Since background microorganisms would be detected in the air and, in an *a priori* effort to put them in some context, we sampled a non-flow cytometer site within the same research laboratory (preparation bench) and a non-flow cytometry laboratory (clinical laboratory) that handles numerous human bacterial pathogens using standard diagnostic microbiological techniques and biosafety risk management procedures.

Flow cytometer equipment and reagents. An Attune NxT (ThermoFisher Scientific, Eugene, OR) acoustic-assisted flow cytometer was the focus of these air sampling investigations. The instrument uses acoustic radiation pressure to align particles in the center of a sample stream. This pre-focused stream is then injected into the sheath stream, which supplies an additional conventional hydrodynamic pressure to the sample. The instrument uses a sheath fluid branded "focusing fluid" that hydrodynamically focuses samples just prior to analysis. The focusing fluid is a proprietary mix of reagents including an unspecified broad spectrum antimicrobial agent (personal communication M. Ward, ThermoFisher, Eugene OR, USA).

Workflow. The air sampling study was conducted over a one month period in which the research laboratory was intensively used by up to nine people at one time during office hours, often with both flow cytometers in use at once. Each flow

cytometer procedure was staffed by one cytometer operator and another scientist preparing bacterial suspensions for FAST and other cytometer assays, plus at least one of the above authors engaged in the air sampling procedure. The majority of flow cytometer experiments analyzed the *K. pneumoniae* isolates as previously reported[13]. The other two species analyzed were *S. pneumoniae* and *B. thailandensis*. The clinical laboratory was staffed between 7.30am and 9pm by up to 20 people, with 1–3 people per side of each laboratory bench, conducting predominantly manual procedures with liquid and solid bacterial cultures. While clinical specimens were opened and blood cultures were subcultured in a class two biosafety cabinet, the majority of bacteriologic procedures were performed on the open bench in accordance with clinical laboratory safety policy[6].

Air sampling. Air sampling was performed with a compact impinger air sampler (MAS-100 Eco, EMD Millipore, Merck) that drew a defined volume of room air over an agar plate positioned in the air sampling unit under the air-permeable lid. Every air sampling in this study was performed at a rate of 100 L per minute for 10 minutes. This is the method used in clean room and operating theatre air quality assessment in government health settings in Western Australia. The device used an Anderson sampler principle to draw air at a constant rate pre-set by the operator onto a 90 mm diameter Petri dish containing agar culture medium, after an initial timed delay to allow the operator to withdraw from its vicinity. The lid surfaces were cleaned with 70% isopropyl alcohol before and after each use in accordance with the manufacturer's instructions.

Two types of media were used; 5% horse blood agar (HBA) and MacConkey agar (MAC) (both supplied by PathWest Laboratory Medicine WA, Mt Claremont WA, Australia). HBA was included as a non-selective medium intended to allow the growth of any of the three target bacteria. MAC was included to minimize the growth of background non-target microorganisms (predicted to be mostly fungi or Gram positive bacteria) while still allowing the growth of *B. thailandensis* or *K. pneumoniae*. Samples taken on HBA while *S. pneumoniae* was being used in the research laboratory were incubated in the presence of 5% CO_2. MAC plates were never incubated in CO_2. All plates were incubated aerobically at 35°C. Colony forming units on both types of solid media were recorded after 24 hr incubation and expressed as CFU/1000 L air. Positive growth controls to confirm the ability of each of the three target bacteria to grow on the media under the chosen incubation conditions were performed.

Air was sampled for identical periods, volumes and locations, onto both agar media on each occasion. Air sampling was conducted before, during and at the end of a day to give a range of times reflecting different levels of research laboratory use and occupation. The same pattern of sampling was conducted for comparison in the clinical bacteriology laboratory. Two main sampling sites were used in the research laboratory (see sampling sites 1 and 2, Supplementary Figure 1). The second of these; sample site 2, was immediately adjacent to the sample introduction port of the flow cytometer between the cytometer and its operator. For comparison, sampling site 1 was on the preparation bench behind the operator approximately 2.5 m from the flow cytometer where 1 ml samples from bacterial cultures (generally 20–30 ml in trypticase soya broth or Mueller Hinton broth) were washed by centrifugation, mixed, diluted and further handled prior to analysis on the flow cytometer. In general, samples analyzed on the cytometer contained approximately 10^6 bacteria/ml or less. During data acquisition for FAST assays, up to 12 samples were analyzed per bacterium. Sample acquisition halted after 1–3 minutes, and each FAST sample was acquired in technical triplicate.

Air sampling was conducted at two sites in the clinical laboratory; the open bench where wound swabs were plated directly onto agar, and adjacent to the rotary plating device used to inoculate agar plates with bacterial suspensions for disk diffusion antimicrobial susceptibility tests (see sites 4 and 5, Supplementary Figure 1).

In the research laboratory, further sets of air samples were collected on multiple occasions after a detergent and ethyl alcohol interventional clean of all laboratory surfaces, and replacement of laboratory gowns. Interventional cleaning was performed late on a Friday and post-clean air samples were acquired beginning the following Monday morning. Cleaning practices in the clinical laboratory were not changed during the course of this study.

Equipment and bench surfaces. On completion of repeated air sampling over one month, surface swabs were collected from the external flow cytometer housing above, below and around the flow sample introduction port, the nearby open bench and the preparation bench opposite before any interventional cleaning had occurred. Swabs (peel pouch Dryswab™ catalogue number MW112; Medical Wire and Equipment Company, Corsham, Wiltshire, England) were immersed in sterile 0.9% normal saline before rubbing vigorously over a 2.5 cm diameter circular area for 1.5 minutes in a spiral motion beginning at the centre and rotating the swab continuously. Swabs were inoculated onto HBA first and then MAC using opposite sides of the swab for each plate. Swabs were collected at each site on at least two occasions. Incubation conditions were as previously described for air samples. Colony forming units on both types of solid media were recorded after 24 hr incubation at 35°C and expressed as CFU/swab.

Bacterial identification. All bacteria growing on MAC were identified using the clinical bacteriology laboratory's identification protocol. In short, after macroscopic examination and discretionary Gram stain analysis, definitive identification was by matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF) from an extended mass spectrometer profile library applying thresholds of 1.80 and 2.00 as the acceptable lower limits for genus and species level identifications, respectively. Initial borderline identification was repeated

on the same sample on the stainless steel target with repeated MALDI-TOF analysis. Potential clinically significant isolates known to be problematic by MALDI-TOF or showing borderline acceptable identification were then subject to supplementary methods such as substrate utilisation panels (e.g. API20E, BioMerieux, France). Microbial growth on HBA was frequently heavy making it difficult to isolate and identify all colonies. Consequently, only the six commonest colony types were identified plus any that resembled the three target bacteria being interrogated by flow cytometry. Due to the selective nature of MAC, fewer colonies occurred and attempts were made to identify all isolates growing on MAC.

Statistical analysis. Column statistics, Chi squared test and non-parametric tests (Mann-Whitney U test) were conducted with Prism statistical software (Prism 6.0, GraphPad, San Diego, CA).

Results

Details of each sample including laboratory location, sampling site, date, time and laboratory activity status are shown in the supplementary data file. In the research laboratory, the bacteria isolated from over 50,000 L of air sampled before, during and after flow cytometer operation did not yield a single cultured colony of *K. pneumoniae, S. pneumoniae* or *B. thailandensis* (Table 1A, Table 1B). None of these species was recovered from either medium during the air sampling period, nor were they recovered from surface swabs (data not shown) taken above, below and around the flow cytometer sample introduction port or other research laboratory surfaces. Bacterial suspensions grown on HBA for experiments conducted on the flow cytometer remained culturable throughout the air sampling period. Positive growth controls confirmed the ability of each of the three target bacteria to grow on the media under the chosen incubation conditions. The bacteria we isolated from air samples in both laboratories at 1-189 CFU/1000 L air were predominantly commensal skin organisms such as coagulase-negative staphylococci and micrococci. The Gram negative bacteria we isolated on either medium by air sampling in both laboratories were environmental species. In the clinical laboratory, Gram negative bacteria were not amongst the six most common colony types detected on HBA. They were detected on MAC at concentrations of 1-5 CFU/1000 L air, much lower than the concentrations of Gram positive bacteria detected on HBA.

Total bacterial air counts increased during the day from <10 to 80–90 CFU/1000 L air both adjacent to the flow cytometer and at the nearby preparation bench (Figure 1). Airborne bacterial counts (CFU/1000 L air) in the clinical bacteriology laboratory spanned a wider range of values but not a significantly different distribution (Table 2). There was a significant fall in bacterial counts from air samples collected adjacent to the flow cytometer (54 CFU/1000 L air falling to 15.5 CFU/1000 L air) after interventional cleaning of research laboratory surfaces and replacement of gowns (Figure 2).

Table 1A. Identities of the commonest bacterial species isolated on blood agar† from 1000 L air samples, arranged by laboratory location. †sampling onto HBA frequently yielded plates crowded with microbial growth. Only the six commonest colony types were identified plus any that resembled the three target bacteria ‡results from both sampling sites in the clinical laboratory were pooled for this table

		Bacteria identified from blood agar		
Laboratory location	**Sampling site**	**Gram positive cocci**	**Gram negative bacilli**	**Gram positive bacilli**
Research laboratory	Sample introduction port of the acoustic-assisted flow cytometer	*Staphylococcus caprae, S. epidermidis, S. haemolyticus, S. hominis, S. saprophyticus, S. warneri, Micrococcus luteus, Massilia timonae*	*Acinetobacter lwoffi, Pseudomonas oryzihabitans, Pantoea agglomerans*	*Bacillus licheniformis, B. megaterium*
	Preparation bench	*S. epidermidis, S. warneri, S. caprae, S. xylosus, S. equis, S. haemolyticus, S. hominis, S. saprophyticus, M. luteus*	*P. oryzihabitans, P. luteola*	*Agrobacterium radiobacter*
Clinical laboratory	Open benches‡	*S. epidermidis, S. haemolyticus, S. saprophyticus, S. warneri, S. caprae, M. luteus*	(not present among identified bacteria)†	(not present among identified bacteria)

Table 1B. Incidence of isolation of all Gram negative bacterial species sampled onto MacConkey agar and identified in 1000 L air samples according to laboratory location. ‡results from both sampling sites in the clinical laboratory were pooled for this table

	Laboratory location and sampling site		
	Research laboratory		Clinical laboratory
Gram negative bacteria identified from MacConkey agar	**Acoustic-assisted flow cytometer**	**Preparation bench**	**Open benches‡**
Pseudomonas luteola	0	1	3
P. oryzihabitans	4	3	0
Unidentifiable	1	0	3
No bacterial growth	17	14	13

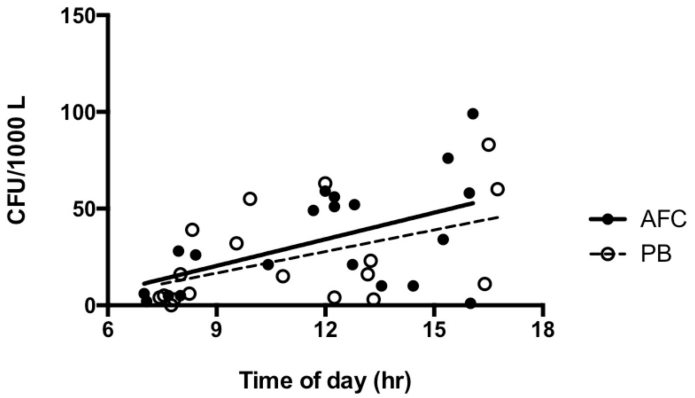

Figure 1. Airborne bacterial count detected on blood agar increases in the research laboratory over the course of the day, both beside the acoustic-assisted flow cytometer (AFC) and at the preparation bench (PB) (respective regression lines shown).

Table 2. Bacterial counts (CFU/ 1000 L air) recovered from air sampled onto blood agar in the Research Laboratory or the Clinical Laboratory. †'In use' refers to cytometer in operation for bacterial analysis. ‡results from both sampling sites in the clinical laboratory were pooled for this table

Laboratory location	Sampling site	Number of samples	Number of CFU/1000 L air on blood agar		
			Lower limit	Median	Upper limit
Research laboratory	Acoustic flow cytometer, not in use†	9	0	5	85
	Acoustic flow cytometer, in use	10	10	54	99
	Acoustic flow cytometer, post clean	4	1	15.5	21
	Preparation bench	20	0	15.5	83
Clinical laboratory	Open benches‡	14	0	33.5	189
All locations	All sites	57	0	21	189

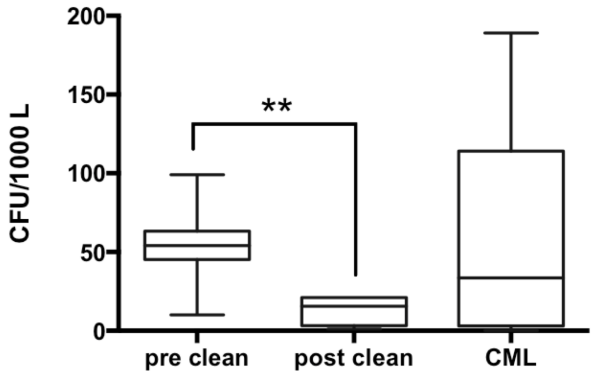

Figure 2. Airborne bacterial count close to acoustic flow cytometer sample introduction port during use, before (pre clean) and after research laboratory cleaning (post clean), compared with samples from the clinical microbiology laboratory (CML) where no interventional cleaning was undertaken. Pre clean research laboratory and post-clean median counts in close proximity to flow cytometer sample introduction port = 54 and 15.5 CFU/ 1000 L, U = 2.5, **p = 0.0090.

Discussion

The stated aim of the present study was to assess the potential risk of personnel exposure to viable bacteria aerosolized during analysis of unfixed bacterial suspensions with an acoustic-assisted flow cytometer, and taking note of current concepts of laboratory safety[14,15], determine mitigation measures to reduce any risk posed by the future introduction of this flow cytometer method into a clinical laboratory. The absence in research laboratory air samples of any of the three bacterial species analyzed with the flow cytometer over a month of intensive laboratory work indicates that these three species represent a low aerosolization risk during the procedures we currently use. None of the analyses involve fluorescence-activated cell sorting, a process known to potentially generate bioaerosols[11,12,16], and for which standard methods of detection have been developed[11]. The absence of any of these three species on the external surfaces of the flow cytometer is further evidence that the cytometer sample introduction port does not generate bacterial aerosols and that basic procedures of wiping surfaces with 70% ethyl alcohol are sufficient to adequately maintain a safe working environment. While our data applies only to the three species we assessed and does not necessarily predict the likelihood of other bacteria becoming airborne during flow cytometer procedures, it is likely that the occupational risks of handling other bacterial species with the same physical properties can be assessed by this approach. Clinical laboratories are subject to robust occupational health and safety practices in order to render them safe places to work. By comparing the viable bacterial content of the two laboratories we aimed to establish that the use of the flow cytometer for bacterial analysis would not contribute an additional bacterial load when this equipment is used in future in a clinical service laboratory.

The dominance of skin commensal and environmental species, which has been reported before[17,18], raises wider issues of industrial hygiene which have not been actively investigated in clinical bacteriology laboratories handling class two pathogens. Our air sampling data indicate that viable airborne bacteria are common and may reach high concentrations of colony forming units during peak laboratory working hours. These data do not distinguish between skin bacteria shed by laboratory staff and disturbance of those same species resting on inanimate surfaces, as these issues were beyond the scope of our investigation. Nevertheless, the reduction in airborne bacterial counts documented after a thorough cleaning of research laboratory surfaces and replacement of laboratory gowns suggests that dust particles may be a contributory factor. These sources may be a lesser concern in a clinical bacteriology laboratory, but in a research laboratory developing novel methods of analyzing bacteria, airborne bacteria represent a potential source of media and equipment contamination that need to be brought under control. Contamination of flow cytometer focusing, wash or other fluids, even with non-biological particles, is a source of background noise and needs to be avoided for optimal results. For this reason, bacterial flow cytometer analyses demand some of the contamination control discipline exercised in molecular diagnostic laboratories. The contamination control measures incorporated into FAST procedures include 0.1 µm filtration of all fluids, flow cytometer analysis of suspending fluids for background particulate noise prior to bacterial analyses, sodium hypochlorite treatment of all flow cytometer effluent, and housing one of the acoustic flow cytometers inside a non-operating class two biosafety cabinet. Use of the non-operating cabinet gives the benefit of a physical barrier between the sample introduction port and the operator. Unfortunately, coarse vibration associated with safety cabinet operation may interfere with the accuracy of flow cytometer data capture. Housing the flow cytometer inside a static bio-bubble of suitable biocontainment level will cause less interference from vibration, when analyzing bacterial species requiring a higher biosafety containment level.

This study showed that use of an acoustic-assisted flow cytometer for bacterial analysis over extended periods did not contribute detectable concentrations of test bacteria to the population of bacteria we cultured from air samples collected in a research laboratory environment. Further, the majority of bacteria cultured were skin commensal and environmental bacteria, presumed to have been shed and dispersed or distributed in laboratory air by personnel movements during routine laboratory operation. The concentrations of airborne bacteria detected in the research laboratory were comparable with those detected in a nearby clinical laboratory on the same biomedical campus and were significantly reduced after cleaning measures were introduced in the research laboratory.

The limitations of this study include the relatively small number of samples, the short duration of the observations collected over the course of about one month and the paucity of data available for comparison.

We undertook this work as part of a risk assessment of the hazards posed by analysing unfixed bacteria by flow cytometric methods. None of the bacteria being investigated by flow cytometry were detected in air samples, irrespective of whether the cytometers were in use. Furthermore, the levels of airborne

bacteria detected in the research laboratory were lower than those detected in a large clinical bacteriology laboratory located in an adjacent building on the same campus. We conclude that our flow cytometric analyses of unfixed suspensions of *K. pneumoniae, B. thailandensis* and *S. pneumoniae* do not pose a risk to cytometer operators or other personnel in the laboratory but caution against extrapolation of our results to other bacteria and/or different flow cytometric experimental procedures.

Data availability

The data supporting the findings reported in this study have been uploaded to OSF: osf.io/z8uka[19].

Dataset 1: FAST project bacterial air sampling. Quantitative air sampling data, times, locations, corresponding laboratory activities and qualitative bacteriologic identification results.

Datasets 2 – 6: FAST air sampling Table 1A, Table 1B, and Table 2, and Figure 1 and Figure 2. Analysis of bacterial air sampling during use of acoustic-assisted flow cytometer for bacterial analysis and control settings.

Competing interests

Dr CF Carson is employed by the University of Western Australia and Dr TJJ Inglis is employed by the University of Western Australia and the state pathology service provider, PathWest Laboratory Medicine WA.

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Supplementary material

Supplementary Figure 1. Floor plan of both laboratories used in this study drawn to the same relative scale: A. research laboratory and B. clinical laboratory. Numbered locations: 1. preparation bench, 2. acoustic-assisted flow cytometer, 3. additional bench location used for air sampling, 4. clinical laboratory culture examination bench, 5. Clinical laboratory antimicrobial susceptibility test bench. Biosafety cabinets indicated by diagonal hatched boxes.

Click here to access the data.

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We would like to thank the authors for taking in consideration our comments in their new version.

In this second version, the introduction still remains unclear whether the authors are focussing on “natural” aerosols generated by laboratory practices (ref 9 and 10) or aerosols generated by hydrofocussed flow cytometer (ref 11 and 12). In order to point out the exact purpose of this study and to avoid misunderstanding, the authors should clarify more. Definitions for each of the phenomenom should be given.

In our first reviewing, the authors were asked to provide positives controls. Indeed, there is a line wich was added in the text mentioning that positive controls were performed. In order to validate this work, authors need to provide data of those positives controls.

Competing Interests: No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Reviewer Report 28 March 2018

https://doi.org/10.21956/gatesopenres.13863.r26279

?

Peter A. Lopez
Department of Pathology, New York University School of Medicine, New York, NY, USA

The basic premise of the study, to show that in routine operation the Attune flow cytometer does not release bacteria of the type specified in the environment, seems to be addressed, although sub-optimally in this reviewer's opinion.The author's observation could still be attributed to anti-microbials in the Attune operating buffer.

The authors use of the clinical laboratory as a control is still questioned.

Sadly, Supplementary Figure 1 does little to clarify the laboratory setting for the reader.. Even though lab area is described in the text, no scale is included in the lab layout drawing. The reader can not discerne if there are open areas or walls of shelving that might impede air flow. In addition, information about the facility airflow (air register placement in relation to the instrument, total room air changes per hour, filtration of air) were not found by this reviewer.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Flow cytometry technology and utilization

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 20 March 2018

https://doi.org/10.21956/gatesopenres.13863.r26277

Tor Monsen
Department of Clinical Microbiology, Umeå University, Umeå, Sweden

The manuscript has been revised accordingly and has improved. The authors have added significant information that has improved the quality and message of the present manuscript. However i do lack information regarding the number of staff / m3 (or m2) both in for the research laboratory and the research laboratory. The number of bacteria in air correlated to the number of staff /m3 (or m2). In this study one can assume that the ventilation system is "nearly the same", hence the number of bacteria in air is correlated to the number of staff/m3 (or m2). The estimated numbers/m3 (or m2) are important to interpret the results of Figure 2.

Beside this comment I do not have further comments to the present manuscript.
I do recommend the article to be accepted after addition of the requested figures.

Recommended articles to the authors
https://www.tandfonline.com/doi/pdf/10.3155/1047-3289.61.7.732

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3444341/

http://images.biomedsearch.com/25006349/1754-9493-8-27.pdf?AWSAccessKeyId=AKIAIBOKHYOLP4M

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 08 December 2017

https://doi.org/10.21956/gatesopenres.13819.r26119

Peter A. Lopez
Department of Pathology, New York University School of Medicine, New York, NY, USA

Thank you for the opportunity to participate in the review of the manuscript " Air sampling during flow cytometric analysis of unfixed bacterial suspensions; a risk assessment "

I believe the authors were trying to assess the possibility of aerosol generation from bacterial samples run on an acoustically-focused flow cytometric amalyzer. It is not clear if the intent of the paper was to document potential aerosol, or aerosolized viable microbes. This very important distinction would add to the paper's reach. Regardless, their testing methods were not as rigorous as previous publications of a similar nature (which they did include in their references (their reference 11 and 12), and only confirmed information already available -- that analytical cytometers do not generate aerosol during routine operation).
Of note, the authors did not include pertinent references that point to the lack of aerosol generation by analytical flow cytometers (see citations 1&2, added in this review).

While additional comments are made in the body of this review, one critical piece of information that is missing in this study revolves around the fact that flow cytometers, including the Attune, add a buffer to the sample during the analysis. Some buffers, such as the FACSflow buffer marketed by Beckton Dickinson, includes anti-microbial agents in the buffer. The authors should have inquired as to the

potential anti-micobials that may be contained in the focusing buffer utilized in the Attune. Obviously if this buffer contains such additives the question of aerosolized viable microbes was not addressed.

As the Attune line of instrumentation from Thermo Fisher utilizes a new technique for focusing cells as they pass through the instrument known as acoustic focusing, there may be concern that this new modality could introduce aerosolization of sample material. If the authors had utilized published methods of aerosol assessment as outlined in the papers that they referenced, this would have clearly added to the value and reproducible of this risk assessment study.

The abstract itself is not clear and does not work work as a standalone summary of this work. In the abstract the authors they include mention of testing in a research laboratory, as well as a "nearby clinical laboratory" without noting why two different labs were being compared. The authors mention extensively that 1000 liter air samples were evaluated for airborne bacteria, with no rationale given for this volume of sampling. Readers are left to wonder if this is an industry-standard sample size, or alternatively, if this may represent an under sampling.

The study design and technical soundness of the work is questionable since the authors do not use previously published methods, do not provide any information regarding the bacterial concentration of samples run on the cytometer, or how many sample tubes were being run on the cytometer during the testing period. The authors did describe the lab workflow-- number of people in the lab, hours of operation, and types of tests, but this adds little to the information needed to reproduce this work, or to validate the impact of the results. A basic diagram of the lab, to include outlines of instrumentation and benches to scale, room dimensions, and including more detail of air-sampling locations, would have been very informative for this study.

It is this reviewer's opinion that without this basic information this study could not be reproduced by a reader.

Those skilled in the art might have concerns that the authors have different interpretations of widely used acronyms, using flourescence-assisted cell sorting rather that flourescence activated cell sorting (the original definition of FACS), and then building upon this interpretation in their definition of the FAST assay, a test which while referenced, could have been briefly described in more detail for those not familiar with this paper. Also HEPA fitration is defined in the paper as "high efficiency purified air", where the commonly utilized definition is either High-Efficiancy Particulate Air, or High-Efficiency Particulate Arrestor. Lastly, the authors continue this trend to introduce AFC for acoustic-enhanced flow cytometer, an acronym not even used by the instrument manufacturer. While minor, these lapses may raise questions in the reader's mind.

While the conclusions drawn may be accurate-- that the authors saw no bacteria becoming airborne as a result of acoustically-focused flow cytometry in their assay, their methods can be challenged and would be difficult to reproduce.

In light of the points made above, I am sorry to suggest that the current study in its current form should be rejected

References

1. Miller JM, Astles R, Baszler T, Chapin K, Carey R, Garcia L, Gray L, Larone D, Pentella M, Pollock A, Shapiro DS, Weirich E, Wiedbrauk D, Biosafety Blue Ribbon Panel, Centers for Disease Control and Prevention CDC: Guidelines for safe work practices in human and animal medical diagnostic laboratories.

Recommendations of a CDC-convened, Biosafety Blue Ribbon Panel.*MMWR Suppl*. 2012; **61** (1): 1-102
PubMed Abstract
2. Sewell D.L, Bove K.E, Callihan D.R: Protection of Laboratory Workers from Occupationally Acquired Infections. *Approved Guideline—Third Edition (M29-A3)*. 2005.

Is the work clearly and accurately presented and does it cite the current literature?
No

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
No

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Flow cytometry technology and utilization

I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 05 Feb 2018

Timothy Inglis, Queen Elizabeth II Medical Centre, Nedlands, Australia

Reviewer: I believe the authors were trying to assess the possibility of aerosol generation from bacterial samples run on an acoustically-focused flow cytometric amalyzer. It is not clear if the intent of the paper was to document potential aerosol, or aerosolized viable microbes. This very important distinction would add to the paper's reach.

Response

- We have clarified text in the Title, Abstract, Introduction and Discussion to reflect that detection of viable aerosolized target bacteria before, during or after use of the acoustic-assisted flow cytometer was the aim of the study.

Reviewer: Regardless, their testing methods were not as rigorous as previous publications of a similar nature (which they did include in their references (their reference 11 and 12), and only confirmed information already available -- that analytical cytometers do not generate aerosol during routine operation).

- This was a very practical investigation designed to investigate if there was a risk of exposure to aerosolized test bacteria for personnel using the flow cytometer.

Reviewer: Of note, the authors did not include pertinent references that point to the lack of aerosol generation by analytical flow cytometers (see citations 1&2, added in this review).

- These references suggested by the reviewer are relevant and have been inserted: Sewell et al. and Miller et al., in the second last paragraph of the Introduction.

Reviewer: While additional comments are made in the body of this review, one critical piece of information that is missing in this study revolves around the fact that flow cytometers, including the Attune, add a buffer to the sample during the analysis. Some buffers, such as the FACSflow buffer marketed by Beckton Dickinson, includes anti-microbial agents in the buffer. The authors should have inquired as to the potential anti-micobials that may be contained in the focusing buffer utilized in the Attune. Obviously if this buffer contains such additives the question of aerosolized viable microbes was not addressed.

- Inclusion of an antimicrobial agent in the Attune Focusing Fluid has been clarified in the “Flow cytometer equipment and reagents” Methods section. Advice from the manufacturer is that some of the first part of the sample may mix with the sheath fluid but that the bulk of the sample, centered by the focusing forces, would only be exposed to the agent through diffusion. The window of opportunity during which contact between the sample and the sheath fluid could occur by diffusion is between the sample leaving the injector and reaching the laser.

Reviewer: As the Attune line of instrumentation from Thermo Fisher utilizes a new technique for focusing cells as they pass through the instrument known as acoustic focusing, there may be concern that this new modality could introduce aerosolization of sample material. If the authors had utilized published methods of aerosol assessment as outlined in the papers that they referenced, this would have clearly added to the value and reproducible of this risk assessment study.

- This was a practical assessment of the risk posed to personnel using the flow cytometer. Detection of viable aerosolized bacteria was attempted using the same method applied for clean room and operating theatre assessment in Western Australia. The manufacturer of the flow cytometer is confident that the instrument does not generate aerosols under normal working conditions.

Reviewer: The abstract itself is not clear and does not work work as a standalone summary of this work. In the abstract the authors they include mention of testing in a research laboratory, as well as a "nearby clinical laboratory" without noting why two different labs were being compared. The authors mention extensively that 1000 liter air samples were evaluated for airborne bacteria, with no rationale given for this volume of sampling. Readers are left to wonder if this is an industry-standard sample size, or alternatively, if this may represent an under sampling.

- The Abstract has been substantially modified for clarity.
- The inclusion of the nearby clinical laboratory as a sampling site has been more clearly justified. See the additional paragraph under Methods, Laboratory Locations.
- The basis for 1000 L air sample size is provided in the Methods, Air Sampling section.

Reviewer: The study design and technical soundness of the work is questionable since the authors do not use previously published methods, do not provide any information regarding the bacterial concentration of samples run on the cytometer, or how many sample tubes were being run on the cytometer during the testing period. The authors did describe the lab workflow-- number of people in the lab, hours of operation, and types of tests, but this adds little to the information needed to reproduce this work, or to validate the impact of the results. A basic diagram of the lab,

to include outlines of instrumentation and benches to scale, room dimensions, and including more detail of air-sampling locations, would have been very informative for this study.

- The range of bacterial concentrations of samples has been added to the text.
- A schematic of both laboratories has been added to the Supplementary information
- The approximate area and volume of each laboratory has been added to the text.

Reviewer: It is this reviewer's opinion that without this basic information this study could not be reproduced by a reader.

Those skilled in the art might have concerns that the authors have different interpretations of widely used acronyms, using flourescence-assisted cell sorting rather that flourescence activated cell sorting (the original definition of FACS), and then building upon this interpretation in their definition of the FAST assay, a test which while referenced, could have been briefly described in more detail for those not familiar with this paper. Also HEPA fitration is defined in the paper as "high efficiency purified air", where the commonly utilized definition is either High-Efficiancy Particulate Air, or High-Efficiency Particulate Arrestor. Lastly, the authors continue this trend to introduce AFC for acoustic-enhanced flow cytometer, an acronym not even used by the instrument manufacturer. While minor, these lapses may raise questions in the reader's mind.

- Abbreviations have been amended/eliminated and/or harmonized.

Reviewer: While the conclusions drawn may be accurate-- that the authors saw no bacteria becoming airborne as a result of acoustically-focused flow cytometry in their assay, their methods can be challenged and would be difficult to reproduce.

In light of the points made above, I am sorry to suggest that the current study in its current form should be rejected

Response: We thank the reviewer for their careful reading and commentary on this paper.
Major revision has been undertaken and we trust the manuscript is now acceptable for publication.

Competing Interests: No competing interests

Reviewer Report 07 December 2017

https://doi.org/10.21956/gatesopenres.13819.r26113

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Coralie L. Guerin
National Cytometry Platform, Department of Infection and Immunity, Luxembourg Institute of Health, Strassen, Luxembourg
Lea Guyonnet
Luxembourg Institute of Health, Strassen, Luxembourg

In this study, the authors investigated the formation of bioaerosols while analyzing three species of bacteria with an acoustic-enhanced flow cytometer (AFC) in a research laboratory thus assessing the risk for the staff to be exposed to bacteria. The authors sampled room air during one month on daily basis before, during and after acoustic-enhanced flow cytometer use.

There are some major comments concerning this manuscript.

1. Indeed, assess the risk of aerosol generation from samples loaded in cytometer is of interest in the field to evaluate and prevent potential transmission to operator. This risk of exposure is well known for hydrodynamic focusing cell sorter in which pressurization of the sample and jet in air system lead to high generation of aerosol. In the manuscript, authors are using an acoustic focusing cytometer where the sample is aspirated in a Hamilton syringe before being focused in the flow cell through ultrasound. Before showing absence of unfixed bacteria in aerosols around the AFC, authors should first test whether this instrument is generating aerosol by using glow germ for example.

2. The duration and frequencies of air sampling are not clearly mentioned. The authors should present a detailed timetable of the samplings indicating duration and frequencies.

3. The absence of CFU for air sampling/swabbing cannot be interpreted since positive and negative controls are missing. Experiments showing the ability of the three bacteria to grow on media used as positive control and experiments with culture media alone and ‘clean’ air sampling without bacteria loaded into the AFC as negative controls should be presented.

4. Results from swabbing even negative need to be shown with appropriate controls.

5. In the discussion, comparison with hydrodynamic focused flow cytometer is missing.

There are some minor comments concerning this manuscript.

1. Manuscript title does not reflect the aim of the study.

2. Regarding methods, the authors indicates that two AFC and two class II biosafety cabinets are presents in the research lab, do AFC are under those biosafety cabinets?

3. As comparison, the authors used a clinical laboratory that is not using AFC technique. In the manuscript, specification about size, layout and staff per m2 are missing. Specifications of both laboratory should be presented.

4. Is the clinical laboratory equipped with air conditioning and/or HEPA filters? ie same air renewal per hour

5. Concerning air sampling in the clinical laboratory, the authors mention that the sampling location was conducted at the open bench, was it near the operator? In the research lab, two sampling locations were used. Please comment the choice of having only one in the clinical laboratory.

6. Which kind of media was used? FBA, HBA, BA are mentioned in the manuscript. If they are identical, harmonization should be done. If not, please explain differences.

In conclusion, the authors states that the risk for staff to be exposed to the analyzed bacteria is non-existent. The present study could be of interest but detailed information needed for full reproducibility

and appropriate controls to be able to draw a conclusion are missing and do not allow conclusion regarding the study.

Is the work clearly and accurately presented and does it cite the current literature?
No

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Flow cytometry

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Author Response 05 Feb 2018

Timothy Inglis, Queen Elizabeth II Medical Centre, Nedlands, Australia

Reviewer: In this study, the authors investigated the formation of bioaerosols while analyzing three species of bacteria with an acoustic-enhanced flow cytometer (AFC) in a research laboratory thus assessing the risk for the staff to be exposed to bacteria. The authors sampled room air during one month on daily basis before, during and after acoustic-enhanced flow cytometer use.

There are some major comments concerning this manuscript.

1. Indeed, assess the risk of aerosol generation from samples loaded in cytometer is of interest in the field to evaluate and prevent potential transmission to operator. This risk of exposure is well known for hydrodynamic focusing cell sorter in which pressurization of the sample and jet in air system lead to high generation of aerosol. In the manuscript, authors are using an acoustic focusing cytometer where the sample is aspirated in a Hamilton syringe before being focused in the flow cell through ultrasound. Before showing absence of unfixed bacteria in aerosols around the AFC, authors should first test whether this instrument is generating aerosol by using glow germ for example.

- **Response**. The literature is replete with assertions that hydrodynamic flow cytometers without sorting capacity do not generate aerosols but empirical data are hard to find. All the

publications containing data found with Google Scholar using the search phrase "Glo Germ" OR "Glo-Germ" "flow cytometer" OR "flow cytometry" relate to cell sorting. We were unable to find any papers using Glo-Germ to evaluate aerosol generation on a hydrodynamic flow cytometer without cell sorting.

- The Glo Germ suggestion has some merit although it is important to consider that in terms of their aerosolization potential, all bacteria are not born equal. Numerous studies demonstrate that different bacteria are variably prone to aerosolization (Parker et al., Amer Rev Resp Dis 1983; Angenent et al., PNAS 2005; Gauthier-Levesque et al., BMC Res Notes 2016; Perrott et al., J Appl Microbiol 2017; Veillette et al., J Aerosol Sci 2018). We sought to determine if viable *K. pneumoniae, S. pneumoniae* or *B. thailandensis* from our assays were entering the air in and around the analytical instrument. Glo Germ are fluorescent 5-µm melamine copolymer resin beads used as bacterial facsimiles. Whether these adequately represent the aerosolization potential of different bacteria remains unclear.
- Our study was deliberately a very practical one designed to identify whether viable bacteria could be detected in the air around the acoustic flow cytometer sample introduction port and the operator during use. We sought to evaluate the risk these procedures posed to laboratory personnel. We appreciated that background microorganisms would be detected in the air and, in an *a priori* effort to put them in some context, sampled a non-flow cytometer site within the same laboratory (preparation bench) and a non-flow cytometry laboratory (clinical laboratory) that handles numerous human pathogens using standard diagnostic microbiological techniques.

2. Reviewers: The duration and frequencies of air sampling are not clearly mentioned. The authors should present a detailed timetable of the samplings indicating duration and frequencies.

- **Response**. The duration of sampling was identical in each run; 100 L per minute for 10 minutes.
- All the dates, times and frequencies of sampling are shown in the detailed data set uploaded to Open Science Forum at the doi provided with this article.

3. Reviewers. The absence of CFU for air sampling/swabbing cannot be interpreted since positive and negative controls are missing. Experiments showing the ability of the three bacteria to grow on media used as positive control and experiments with culture media alone and ‘clean’ air sampling without bacteria loaded into the AFC as negative controls should be presented.

- **Response**. The following text has been added to the Air Sampling section to clarify these points:

Two types of media were used: 5% horse blood agar (HBA) and MacConkey agar (MAC) (both supplied by PathWest Laboratory Medicine WA, Mt Claremont WA, Australia). HBA was included as a non-selective medium intended to allow the growth of any of the three target bacteria. MAC was included to minimize the growth of background non-target microorganisms (predicted to be mostly fungi or Gram positive bacteria) while still allowing the growth of *B. thailandensis* or *K. pneumoniae*. Samples taken on HBA while *S. pneumoniae* was being used in the research laboratory were incubated in the presence of 5% CO_2. MAC plates were never incubated in CO_2. All plates were incubated aerobically at 35°C. Colony forming units on both types of solid media were recorded after 24 hr incubation and expressed as CFU/1000 L air. Positive growth controls to confirm the ability of each of the three target bacteria to grow on the media under the chosen incubation conditions were performed.

4. Reviewers. Results from swabbing even negative need to be shown with appropriate controls.

- **Response**. Positive growth controls demonstrated that the target bacteria were able to grow on the media. This information has been added to the text. All swab results are shown in the accompanying data set.

5. Reviewers. In the discussion, comparison with hydrodynamic focused flow cytometer is missing.

- **Response**. Comparison with hydrodynamic systems is difficult since as mentioned above at point 1, while the literature is replete with assertions that hydrodynamic flow cytometers without sorting capacity do not generate aerosols, empirical data are hard to find.

Reviewers. There are some minor comments concerning this manuscript.

1.Manuscript title does not reflect the aim of the study.

- **Response**. The title has been amended to more accurately reflect the revised content.

2. Regarding methods, the authors indicates that two AFC and two class II biosafety cabinets are presents in the research lab, do AFC are under those biosafety cabinets?

- **Response**. This has been clarified in the manuscript:One of the flow cytometers is housed and used in a biosafety cabinet while the other one, the focus of this study, is on the open bench.

3. As comparison, the authors used a clinical laboratory that is not using AFC technique. In the manuscript, specification about size, layout and staff per m2 are missing. Specifications of both laboratory should be presented.

- **Response**. This information has been added to the manuscript.

4. Is the clinical laboratory equipped with air conditioning and/or HEPA filters? ie same air renewal per hour

- **Response**. No information was available about the air renewal per hour. The following has been added to the manuscript:Both laboratories were air-conditioned and equipped with high efficiency particulate air filtration on external air outlets. Detailed information about the number of complete air exchanges per hour was not available for either location.

5. Concerning air sampling in the clinical laboratory, the authors mention that the sampling location was conducted at the open bench, was it near the operator? In the research lab, two sampling locations were used. Please comment the choice of having only one in the clinical laboratory.

- **Response**. Two sites were sampled in both laboratories. Both sampling sites in both labs were taken near the personnel. A third site was occasionally sampled in the research laboratory. Details of all the sample sites are shown in the supplementary material.

6. Which kind of media was used? FBA, HBA, BA are mentioned in the manuscript. If they are identical, harmonization should be done. If not, please explain differences.

- **Response**. Abbreviations have been amended/eliminated and/or harmonized.

Reviewers. In conclusion, the authors states that the risk for staff to be exposed to the analyzed bacteria is non-existent. The present study could be of interest but detailed information needed for full reproducibility and appropriate controls to be able to draw a conclusion are missing and do not allow conclusion regarding the study.

We appreciate the reviewers' expert guidance on how to improve the presentation of our observations.

Competing Interests: No competing interests

Author Response 05 Feb 2018

Timothy Inglis, Queen Elizabeth II Medical Centre, Nedlands, Australia

We have addressed the reviewers' comments and recommendations in an amended version of this manuscript, that more clearly states the purpose of our study, and the need to compare with a clinical laboratory environment. Additional data has been provided including a supplementary figure showing the respective laboratory floor plans.

We appreciate the reviewers' expert guidance on how to improve the presentation of our observations.

Competing Interests: No competing interests with the reviewers.

Reviewer Report 27 November 2017

https://doi.org/10.21956/gatesopenres.13819.r26069

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Tor Monsen
Department of Clinical Microbiology, Umeå University, Umeå, Sweden

Many thanks for the opportunity to review the manuscript entitled “Air sampling during flow cytometric analysis of unfixed bacterial suspensions; a risk assessment” by Christine F Carson *et al*.

The topic of the study is important in clinical laboratories regarding the risk for the staff to be exposed to harmful bacteria during their daily work. In this case risk for exposure to harmful bacteria during antimicrobial susceptibility test performed using a flow cell cytometric analysis. The method using “flow cytometric analysis” (FCA) is a relative new technique used in clinical laboratories and an expanding method I clinical microbiology.

I do have some major and minor comment to the present manuscript that has to be taken in consideration prior to acceptance of the manuscript.

1. The title of the manuscript do not clearly describe the intention of the present study. I would suggest that the title adjusted or rewritten to the message of the the present study.

2. Abstract: lack of “Aim(s)” in the text.

3. The authors includes a clinical laboratory (CL) that do not use the FCA technique. However the bacterial concentration in air is measured and compared with that in the research laboratory using the FCA technique. However the design of the two laboratories (research- and clinical laboratory) are very different in construction and size (square meters) and if compared the number of staff/m2 should be presented.

   The clinical laboratory was included just to compare the distribution of bacteria in air with air the

research laboratory which is of secondary interest as a risk assessment since the FCA instrument was not used in the clinical laboratory.

4. Regarding the research laboratory information regarding the FCA should be included in the text whether the instrument is standing on a bench or within a safety cabinet? Closed cabinet? The instrument location is important as information of the ventilation (air replaced XY times/hours). The information is important in order to valuate the results of the present study.

5. Methods: lack information when the measurement were done. At fixed times or random times both in research- and clinical laboratory? The bacterial concentration in air is very closely associated to the number of staff at work as each person is calculated to release 5 CFU/bacteria per second to the environment.

6. The concentration of bacteria used in the FCA lacks (CFU/mL). The risk for environment contamination would probable increase at higher bacterial concentrationsversus low bacterial concentrations. Such comment should be discussed in the section discussion.

7. Section “Air sampling” Two types of media were used: 5% horse blood....
I do recommend that this sentence is rewritten to pinpoint that culture of bacteria were done using 5% horse blood agar …. Information lacks for the supplier of the agar plates and if the MacConkey agar plates were incubated in CO2? Especially if searching for S. pneumoniae.

8. Information of the supplier of the cotton swabs lacks as the size of the area that was swabbed by the cotton swabs? Bacterial content in the swabs is probably related to the area swabbed. Was the collection of specimens standardized?

9. MALDITOF. Definition of the “cut off” for correct / acceptable species identification lacks.

10. Headings of Table 1A, 1B, Table 2 has to be improved. Information regarding from which agar plates used should be presented in the section methods and omitted in the table headings.
Table 1A, “Preparation bench” are “S. warneri, S. caprae” and “S. xylosis, S. equus???” on the same lane and not consistent to the presentation of other species.
Table 1B could be omitted since it does not contribute to the message in the study.

11. Figure 1, text has to be improved. Here, the comment “rises” should be omitted in text. The abbreviations “AFC” and “PB” should explained in the figure text.
Figure 2, Figure text has to be rewritten, I recommend that either one of the terms “during use” or “before pre clean” is deleted or rewritten as “during use (pre-clean). There are no data presented “during use”, only pre-clean (=during use?) and post clean. Also, when (minutes) after cleaning was the specimens collected, this information should be included in the section “Material and methods”

12. The inclusion of a “clinical” laboratory” does contribute to the risk analysis for exposure of potential pathogens to the environment. The inclusion of av microbiological laboratory only serve as “control” to the microorganisms present in the research laboratory (FCA). The bacteria in the environment is as expected however gramnegative and grampositive bacteria were only present in the research unit which might need to be commented in the section “Discussion”.

13. Conclusion. The authors state that FCA do not pose a risk for FCA staff to pose a risk for FCA staff to be exposed to K pneumoniae, B thailandensis and S pneumoniae. I partly agree to the conclusion based on the results from the present study. The present study is small and further studies are warranted to confirm the present results.

Conclusion: The risk for staff exposed to pathogen microorganisms during work is an important issue which is examined in the present study. Overall few studies are found regarding this subject. The present study is well done but more information has to included in text, see comments above.

I recommend that the data from the clinical laboratory is excluded or put in as a text in the manuscript according the aim of the present study as I hardly find it to contribute to the risk assessment associated to the flow cytometry analysis.

The article could be accepted after a major revision

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 05 Feb 2018

Timothy Inglis, Queen Elizabeth II Medical Centre, Nedlands, Australia

Reviewer: The topic of the study is important in clinical laboratories regarding the risk for the staff to be exposed to harmful bacteria during their daily work. In this case risk for exposure to harmful bacteria during antimicrobial susceptibility test performed using a flow cell cytometric analysis. The method using “flow cytometric analysis” (FCA) is a relative new technique used in clinical laboratories and an expanding method I clinical microbiology.

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1. The title of the manuscript do not clearly describe the intention of the present study. I would suggest that the title adjusted or rewritten to the message of the the present study.

- **Response**.The title has been amended to more accurately reflect the revised content.

2. Abstract: lack of “Aim(s)” in the text.

- **Response**. We have clarified text in the Title, Abstract, Introduction and Discussion to reflect that detection of viable aerosolized target bacteria before, during or after use of the acoustic-assisted flow cytometer as the aim of the study.

3. The authors includes a clinical laboratory (CL) that do not use the FCA technique. However the bacterial concentration in air is measured and compared with that in the research laboratory using the FCA technique. However the design of the two laboratories (research- and clinical laboratory) are very different in construction and size (square meters) and if compared the number of staff/m2 should be presented.The clinical laboratory was included just to compare the distribution of bacteria in air with air the research laboratory which is of secondary interest as a risk assessment since the FCA instrument was not used in the clinical laboratory.

- **Response.** The inclusion of the nearby clinical laboratory as a sampling site has been more clearly justified. The following text has been added to the Methods, Laboratory Locations section:

  The clinical laboratory was included for comparative purposes. While the study aimed to determine if aerosolized viable target bacteria (*B. thailandensis, K. pneumoniae* and *S. pneumoniae*) could be detected in air in the research laboratory, the likelihood was that other microorganisms, commonly present in indoor air, would be detected. Published data regarding the range and concentration of bacteria present in air in microbiology laboratories, either research or clinical, are scarce. Without data for comparison, data on the viable bacteria detected in air samples from the research laboratory would be entirely without context. Work practices in the clinical laboratory are designed to maintain a safe working environment and the background level of viable bacteria detected in clinical laboratory air could therefore serve as a proxy indicator of the acceptable level of viable bacteria in air in the research laboratory.

  Since background microorganisms would be detected in the air and, in an a priori effort to put them in some context, we sampled a non-flow cytometer site within the same research laboratory (preparation bench) and a non-flow cytometry laboratory (clinical laboratory) that handles numerous human bacterial pathogens using standard diagnostic microbiological techniques and biosafety risk management procedures.

4. Regarding the research laboratory information regarding the FCA should be included in the text whether the instrument is standing on a bench or within a safety cabinet? Closed cabinet? The instrument location is important as information of the ventilation (air replaced XY times/hours). The information is important in order to valuate the results of the present study.

- **Response**. Information about the position of the flow cytometer (on the bench, outside a safety cabinet) has been added. Approximate area and volume of the spaces has also been added. Unfortunately, data on the rate of air replacement were not available for either laboratory location.

5. Methods: lack information when the measurement were done. At fixed times or random times both in research- and clinical laboratory? The bacterial concentration in air is very closely associated to the number of staff at work as each person is calculated to release 5 CFU/bacteria per second to the environment.

- **Response**. All the information about sampling times, sites and laboratory locations is given in the data file uploaded with this manuscript.
- It would be helpful to include the reference that calculates the release of bacteria into air from each person as 5 CFU of bacteria per second. We would be grateful if the reviewer could let us know the reference.

6. The concentration of bacteria used in the FCA lacks (CFU/mL). The risk for environment contamination would probable increase at higher bacterial concentrationsversus low bacterial concentrations. Such comment should be discussed in the section discussion.

- **Response**. This information has been added to the manuscript:

  For comparison, the second sampling site was on the preparation bench behind the operator approximately 2.5 m from the flow cytometer where 1 ml samples from bacterial cultures (generally 20-30 ml in trypticase soya broth or Mueller Hinton broth) were washed by centrifugation, mixed, diluted and further handled prior to analysis on the flow cytometer. In general, samples analyzed on the cytometer contained approximately 10^6 bacteria/ml or less. During data acquisition for FAST assays, up to 12 samples were analyzed per bacterium. Sample acquisition halted after 1-3 minutes, and each FAST sample was acquired in technical triplicate.

7. Section “Air sampling” Two types of media were used: 5% horse blood....I do recommend that this sentence is rewritten to pinpoint that culture of bacteria were done using 5% horse blood agar …. Information lacks for the supplier of the agar plates and if the MacConkey agar plates were incubated in CO2? Especially if searching for S. pneumoniae.

- **Response**. This information has been added to the manuscript:

  Two types of media were used: 5% horse blood agar (HBA) and MacConkey agar (MAC) (both supplied by PathWest Laboratory Medicine WA, Mt Claremont WA, Australia). HBA was included as a non-selective medium intended to allow the growth of any of the three target bacteria. MAC was included to minimize the growth of background non-target microorganisms (predicted to be mostly fungi or Gram positive bacteria) while still allowing the growth of B. thailandensis or K. pneumoniae. Samples taken on HBA while S. pneumoniae was being used in the research laboratory were incubated in the presence of 5% CO_2. MAC plates were never incubated in CO_2. All plates were incubated aerobically at 35°C. Colony forming units on both types of solid media were recorded after 24 hr incubation and expressed as CFU/1000 L air. Positive growth controls to confirm the ability of each of the three target bacteria to grow on the media under the chosen incubation conditions were performed.

8. Information of the supplier of the cotton swabs lacks as the size of the area that was swabbed by the cotton swabs? Bacterial content in the swabs is probably related to the area swabbed. Was the collection of specimens standardized?

- **Response**. Details of the swabs and the collection method have been added:

  Swabs (peel pouch Dryswab™ catalogue number MW112; Medical Wire and Equipment Company, Corsham, Wiltshire, England) were immersed in sterile 0.9% normal saline before rubbing vigorously over a 2.5 cm diameter circular area for 1.5 minutes in a spiral motion beginning at the center and rotating the swab continuously. Swabs were inoculated

onto HBA first and then MAC using opposite sides of the swab for each plate. Swabs were collected at each site on at least two occasions.Incubation conditions were as previously described for air samples. Colony forming units on both types of solid media were recorded after 24 hr incubation and expressed as CFU/swab.

9. MALDITOF. Definition of the “cut off” for correct / acceptable species identification lacks.

- **Response**. The species cut-off for MALDI-TOF was >2.00. The genus level threshold was set at 1.80. This information has been added to the manuscript in the Bacterial identification section.

10. Headings of Table 1A, 1B, Table 2 has to be improved. Information regarding from which agar plates used should be presented in the section methods and omitted in the table headings.Table 1A, “Preparation bench” are “S. warneri, S. caprae” and “S. xylosis, S. equus???” on the same lane and not consistent to the presentation of other species.Table 1B could be omitted since it does not contribute to the message in the study.

- **Response**. Headings and content for all tables have been amended. Table 1B has been retained but clarified.

11. Figure 1, text has to be improved. Here, the comment “rises” should be omitted in text. The abbreviations “AFC” and “PB” should explained in the figure text.Figure 2, Figure text has to be rewritten, I recommend that either one of the terms “during use” or “before pre clean” is deleted or rewritten as “during use (pre-clean). There are no data presented “during use”, only pre-clean (=during use?) and post clean. Also, when (minutes) after cleaning was the specimens collected, this information should be included in the section “Material and methods”

- **Response**. The Figure 1 legend text has been amended and the abbreviations addressed. It now reads:

  Figure 1. Airborne bacterial count detected on blood agar increases in the research lab over the course of the day, both beside the acoustic-assisted flow cytometer (AFC) and at the preparation bench (PB) (respective regression lines shown).

  The Figure 2 legend text has been amended. Previously it was:*Figure 2. Airborne bacterial count close to acoustic flow cytometer sample introduction port during use, before (pre clean) and after research laboratory cleaning Post clean), compared with the clinical microbiology laboratory. Pre clean research laboratory and post-clean median counts in close proximity to flow cytometer sample introduction port = 54 and 15.5 CFU/ 1000L, U = 2.5, p = 0.0090.*

  It now reads:

  Figure 2. Airborne bacterial counts from samples taken close to the acoustic flow cytometer sample introduction port during use, compared to counts from clinical laboratory samples. Data shown are for samples taken in the research laboratory before (pre-clean) and after (post-clean) interventional cleaning compared with samples from the clinical microbiology laboratory (CML) where no interventional cleaning was undertaken. Pre-clean and post-clean median counts from the research laboratory in close proximity to the flow cytometer sample introduction port = 54 and 15.5 CFU/ 1000L, U = 2.5, **p = 0.0090.

- Information about the timing of post-clean sampling has been added to the manuscript.

12. Reviewer. The inclusion of a “clinical” laboratory” does contribute to the risk analysis for exposure of potential pathogens to the environment. The inclusion of av microbiological laboratory only serve as “control” to the microorganisms present in the research laboratory (FCA). The

bacteria in the environment is as expected however gramnegative and grampositive bacteria were only present in the research unit which might need to be commented in the section “Discussion”.

- **Response**. We agree with the reviewer that the inclusion of the clinical laboratory serves only to give some context to the microbial content of research laboratory air. The rationale for inclusion of the clinical laboratory has been expanded and clarified. See the information provided in response to review 2, point 1, bullet point 3.

- Gram negative bacteria were cultured in the air samples taken in the clinical laboratory. Clinical laboratory air samples on blood agar often had heavy growth and only the 6 commonest colony types were selected for identification. Gram negative bacteria did not feature amongst the 6 commonest colony types detected on blood agar (Table 1A). Clinical laboratory air samples on MacConkey agar grew Gram negative bacteria on three occasions. Attempts were made to identify all isolates growing on MacConkey agar but these were unidentifiable (see Table 1B). The text and the tables have been modified to clarify these points.

13. Conclusion. The authors state that FCA do not pose a risk for FCA staff to pose a risk for FCA staff to be exposed to K pneumoniae, B thailandensis and S pneumoniae. I partly agree to the conclusion based on the results from the present study. The present study is small and further studies are warranted to confirm the present results.

- **Response**. The outcomes of this study provided empirical reassurance to us that our current laboratory practices with these organisms do not pose a significant hazard to personnel. We agree that the study is small and that further studies are warranted to confirm and expand the present results.

- A section acknowledging the limitations of this study has been added to the manuscript.

Reviewer: Conclusion: The risk for staff exposed to pathogen microorganisms during work is an important issue which is examined in the present study. Overall few studies are found regarding this subject. The present study is well done but more information has to included in text, see comments above.

- **Response**. A significant quantity of additional information has been added throughout the manuscript.

Reviewer. I recommend that the data from the clinical laboratory is excluded or put in as a text in the manuscript according the aim of the present study as I hardly find it to contribute to the risk assessment associated to the flow cytometry analysis.

- **Response**. The Aim of the study has been clarified and the rationale for including the clinical laboratory as a comparator has been more clearly elaborated. Given the information vacuum around the microbial content of indoor air in microbiology laboratories of either persuasion, clinical or research, we respectfully disagree with the reviewer on the value of retaining the data from the clinical laboratory.

Reviewer. The article could be accepted after a major revision

Response. We have addressed all of this reviewer’s comments as well as all the comments from the other 2 reviews. We believe this constitutes a major revision and that the manuscript should now be acceptable.

We would be grateful if the reviewer could let us know the reference for the 5CFU of bacteria released per person per second.

Competing Interests: No competing interests