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Original Research

Evaluate the Role of *Klebsiella pneumoniae* in an Animal Model for Systemic Analysis of Serum Interleukin [IL-6, IL-18 and IL-23] and Chemokines [CCL-5 and CCL-11]

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Abstract:

Klebsiella pneumoniae is an important opportunistic pathogen associated with implant-related and healthcare-associated infections, where early diagnosis is essential for effective treatment. This study investigated the volatile organic compounds (VOCs) produced by carbapenem-susceptible and carbapenem-resistant K. pneumoniae and evaluated the associated inflammatory response in a murine model of implant-associated infection. Bacterial VOCs were identified using gas chromatography–mass spectrometry (GC–MS), and their dynamic production was monitored over 24 h. A subcutaneous mouse infection model was established to determine serum levels of IL-6, IL-18, IL-23, CCL-5, and CCL-11 using multiplex immunoassays. GC–MS analysis identified several characteristic VOCs, including 2-heptanone, 2-pentene, 3-methylbutanal, benzaldehyde, 3-methylbutyl acetate, methacrolein, mercaptoacetone, and 2-phenylfuran, with three distinct release patterns observed during bacterial growth. Mice with bacteria-implant infections exhibited significantly higher serum concentrations of IL-6, IL-18, IL-23, CCL-5, and CCL-11 than animals with sterile implants or bacterial infection alone ($P < 0.05$), indicating a pronounced inflammatory response associated with bacterial colonization of implants. These findings demonstrate that VOC profiling, combined with inflammatory cytokine and chemokine analysis, provides valuable biomarkers for the early diagnosis and monitoring of K. pneumoniae implant-associated infections and may contribute to improved clinical management and therapeutic intervention.

Keywords: *Klebsiella*, *pneumoniae*, Animal Model, Interleukin, Chemokines.

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1-Introduction

The early identification of infectious diseases, along with the identification of a causative agent to the species level is always of paramount clinical relevance. Furthermore, this is an important task of a clinical microbiology laboratory. The key is that different bacteria strains have different resistance characteristics, both naturally and intrinsically, to antibiotics. Therefore, the most optimal results in an empirical treatment plan can be obtained by specifically addressing the most likely etiological factor. The ability to identify the infectious agent without culturing the microorganism means that antimicrobial treatment will be more focused and therefore, more likely to eradicate the microorganism [1, 2]. Patients are given broad-spectrum antibiotics, mostly carbapenems, during the two days it takes to know the findings of the invasive sample of bronchoalveolar lavage (BAL) specimens needed for microbiological testing of an underlying pathogen. Although rapid antibiotic use is necessary for improved patient outcomes, overuse of antibiotics can give rise to antimicrobial resistance. In 2019, at least one antimicrobial group was resistant to more than 50% of the Escherichia coli and more than 33% of the *Klebsiella pneumoniae* (KPN) isolates [3]. *Klebsiella pneumoniae* have an ability to acquire resistance mechanisms, including the carbapenemases, which break down antibiotics. The creation and use of an organism-specific therapeutic for VAP appears logical; it would be expected to help curb the development of antimicrobial resistance. To achieve this lofty objective, however, a rapid and non-invasive method of diagnosis of VAP is needed, along with finding out what organism is responsible for it. Over the past few years, the range of volatile organic compounds (VOCs) emitted by microorganisms (including harmful bacteria) has been demonstrated. Several of these may be pathogen specific, and some might even serve to discriminate between the various bacteria [4, 5]. Monitoring the concentration profile of bacteria-specific volatile organic compounds (VOC) in the breath of MV patients could be useful to monitor the progress of the infection with the aim of detecting pneumonia early without performing invasive procedures, prescribing the correct antibiotic and promptly and monitoring its efficacy. To accomplish this goal, it is important to also do a model study with reference strains and confirm the results with clinical isolates to ensure accurate identification of metabolites produced by bacteria. In all the studies, bacterial cultures were used to collect headspace samples which were then concentrated on multibed sorption tubes for GC-MS analysis. Under controlled ventilation conditions, the concentration profiles of VOCs have been dynamically monitored at several time points to record the changes over time [6]. The main aim of this research was to analyse the function of *Klebsiella pneumoniae* in an animal model, for systemic examination of serum chemokines (CCL-5 and CCL-11) and interleukins (IL-6, IL-18 and IL-23).

2-Materials and Methods

2-1-Culture of bacteria and processing of samples

A total of 35 male and female patients with ages of 15-25 years were sampled from different hospitals in Babylon area in Iraq, diagnosed to have acute pharyngotonsillitis. Throat swabs were tested for bacteria using various tests. Experimental strains were cultured on blood agar, and incubated at 37 ℃ overnight. After this bacterial suspension was placed in the test tube, a culture media was pipetted into every test tube. In TSB, the curves of K. pneumoniae growth in the presence of supplementary showed. As expected all strains show consistent growth patterns. *Klebsiella pneumoniae* was seen to enter exponential growth phase around 3 hours and growth stabilisation phase around 5 hours. After that, the growth curve data were employed to assess the metabolic kinetics of volatile organic compounds (VOCs) produced by *Klebsiella pneumoniae* by gas chromatography–mass spectrometry. Throughout the studies, no additional processing was required for the samples used for GC-IMS analysis, which included 500

mL of bacterial culture fluid for each sample analysed.

2-2-Investigating the Spectroscopy using GC-MS

The GC-MS Spectroscopic Analysis showed that all of the samples contained methanol. None of the samples were found to be free of any acetone, methanol or ethanol. The analysis was performed using an instrument called GC-MS Agilent 789 that is used to identify the bioactive natural chemical constituents of *Klebsiella pneumoniae*. The parameters of this column are as follows: internal diameter is 30 mm and the outside diameter is 0.25 mm. The film thickness is 0.25 mm. During this trial, the temperature of the furnace was kept constant. We kept the flow rate at 1 mL/min and utilised helium as the carrier gas. In order to prepare the gas chromatography (GC) column effluents for injection into the mass spectrometer (MS), they were heated to 250 degrees Celsius and then transferred into the MS straight from the source. Ionisation can take place at 70 eV as long as the ion source is kept hot, at least at 230 ℃. The mass range of the measured spectra went up to 450 amu. The compounds were exposed using mass spectrum matching to possibly identify chromatographic peak(s). The NIST mass spectrum library is one such source of identification of compounds.

2-3-A model for subcutaneous (SC) implants and infections in mice and other small animals.

Animals used: Mice aged 7-10 weeks. The animals were kept in cages with plenty of food and drink and good ventilation. First, a dose of 4 mg/kg xylazine and 10 mg/kg ketamine was given intravenously into the animals under a sterile bench while they were asleep. After the fur was removed from the implantation sites using a hair trimmer, they were washed with 70% ethanol. Surgical shears and tissue forceps were used to make the three 1cm diameter incisions. Subcutaneous compartments were created by these incisions. Using simple interrupted sutures, the incisions were closed. Not including those that are implanted, within 30 minutes of surgical incisions being closed. Blood samples were taken three weeks after implanting and the animals were then closely monitored.

2-4-Treatment for bleeding and immunisation.

Each mouse was injected with 35.0 g of rPgdA three times (at 14 day intervals) in the subcutaneous tissue, all using the same amount of Inject Alum Adjuvant (Thermo Scientific). The serum samples were collected right before injection and 1 week after the final injection and then frozen at -80°C until further analysis.

2-5-The serum cytokines were assessed. These serum cytokines were evaluated.

Three weeks after the implantations we sacrificed the mice and gave them a good anaesthetic to obtain the blood samples without coagulant. Blood samples were allowed to cool to ambient temperature for half an hour after which the centrifuge was operated for 10 minutes at -4C -1 rpm. The serum was collected after centrifugation and kept at 80 C until further processing. Blood cytokines (IL-6, IL-12, IL-18, CCL-5 and CCL-11) were quantified by the Bio-Plex Pro Mouse Cytokine Assay (Bio-Rad, Munich, Germany).

3-Analysis of data, statistical modelling.

Prior to confirmation by retention time of a reference material, identified chemicals were identified by comparing the obtained spectrum to the NIST 2017 Mass Spectra Library. All the necessary materials for identification of the metabolites found were purchased from Alchem. The peaks with unclear chromatograms were corrected by an experienced GC-MS expert by using a customised procedure in the Shimadzu GCMS Postrun Analysis software. The statistical significance in VOC of the bacterial suspension at different growth time compared to reference TSB medium was calculated using the Statistica 13.3 PL software (TIBCO Software Inc., Tulsa, OK, United States). A non-parametric test for comparing samples from two or more samples of independent observations, the Kruskal-Wallis test was used. P-value < 0.05 was considered statistically significant. The fact that this test is robust against outliers and doesn't assume regularly distributed groups was a major factor in

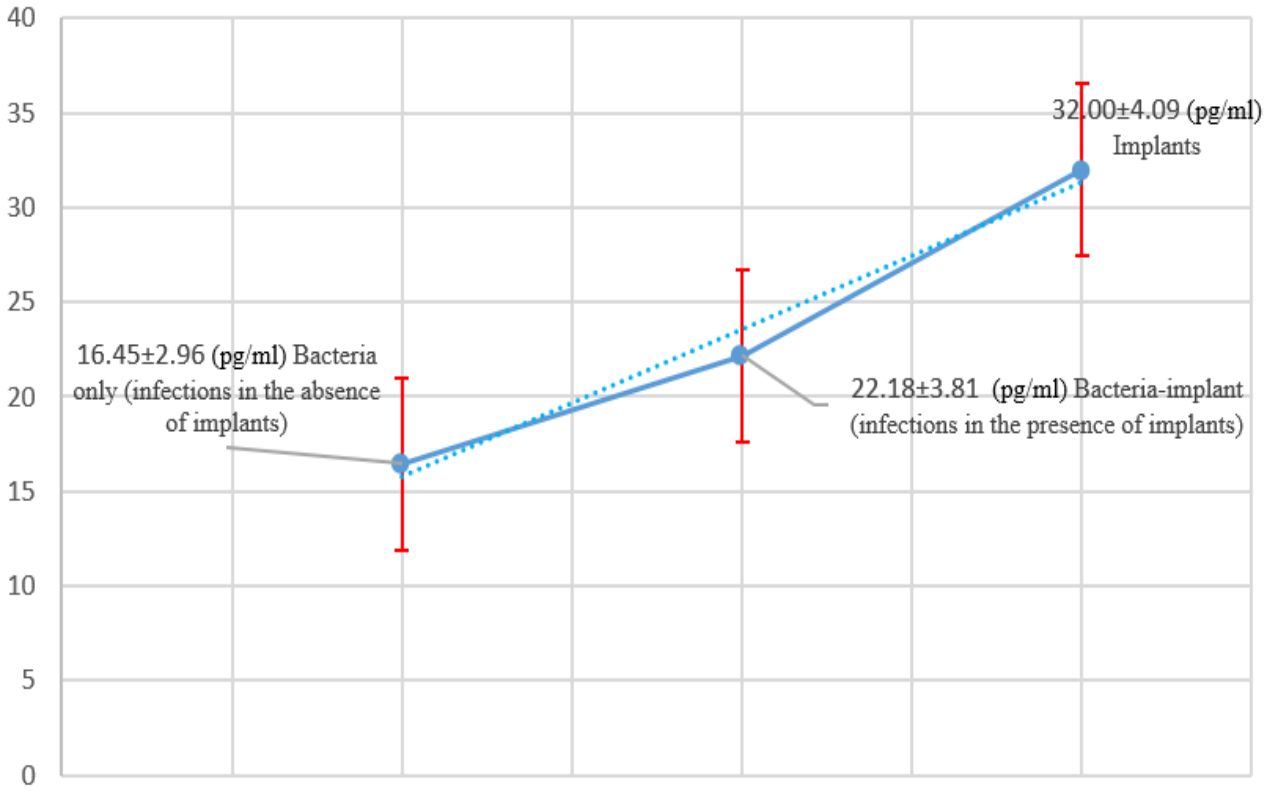
its selection. Because of the new False Discovery Rate adjustment in Statistica 13.3, we used a non-parametric U-Mann-Whitney test to compare abundance of VOCs between only two groups of interest. The peak area was subsequently log transformed and presented for the most important VOCs, using the online program Statistica 13.3 PL and Metaboanalyst 5.0.

4-Results and Discussion

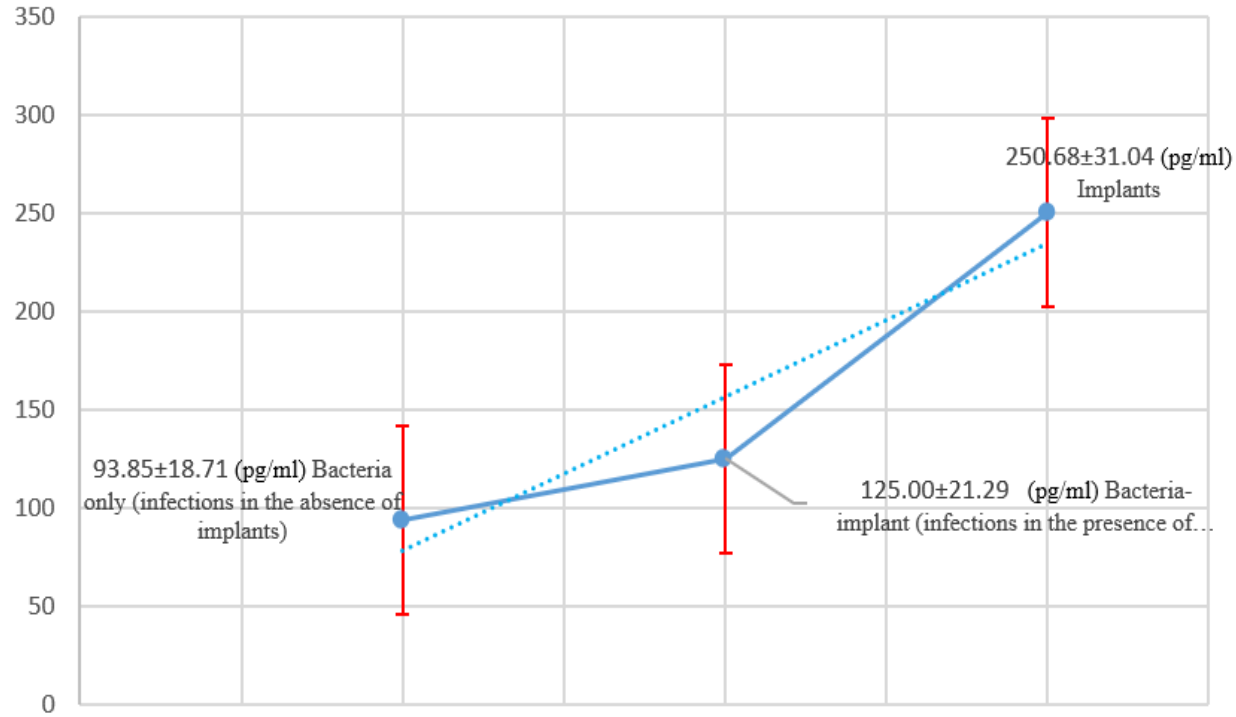
The GC-MS analysis of crude extracts of various species of *Klebsiella pneumoniae* revealed a wide range of compounds. The GC-MS results that revealed the most important and beneficial components of the raw extracts. Nearly every life form made substances that were chemically similar to these. The majority of the molecules that were found belonged to volatile substances, especially 2-Heptanone, 2-Pentene, 2-Amino-2-methyl-1-propanol, 3-Methylbutanal, Benzaldehyde, 3-methyl, 3-Methylbutyl acetate, 1-Propanol, 3-amino, Methacrolein, 2-Butanol, 2-ethyl-5-methylpyrazine, Mercaptoacetone, and 2-phenylfuran. The discussion focuses on different research strategies and methodologies in medical microbiology using the techniques of gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). GC can detect chemical markers (monomer units and metabolites) in microbial cultures; analyses of the fatty acids in cells have been a useful tool for characterising and identifying species. GC-MS can be utilized to analyse airborne organic matter for the chemical makers such as muramic acid, 3-hydroxy acids and ergosterol, which are present in complicated environmental samples. This methodological approach offers a sound basis for association of inhalation of airborne microbial structures and the appearance of symptoms [7, 8] and it is an alternative option to other biological tests for characterisation of these structures. Diagnostic and understanding of the metabolism of microbes in the infected host can be achieved directly by GC-MS analysis of clinical samples, which may have implications for drug design. As long as MS technology continues to improve, researchers in the field of microbiology will be able to make quick progress with GC-MS.

Table 1. Shows the results of the GC-MS analysis of the bioactive chemical compounds produced by *Klebsiella pneumoniae*.

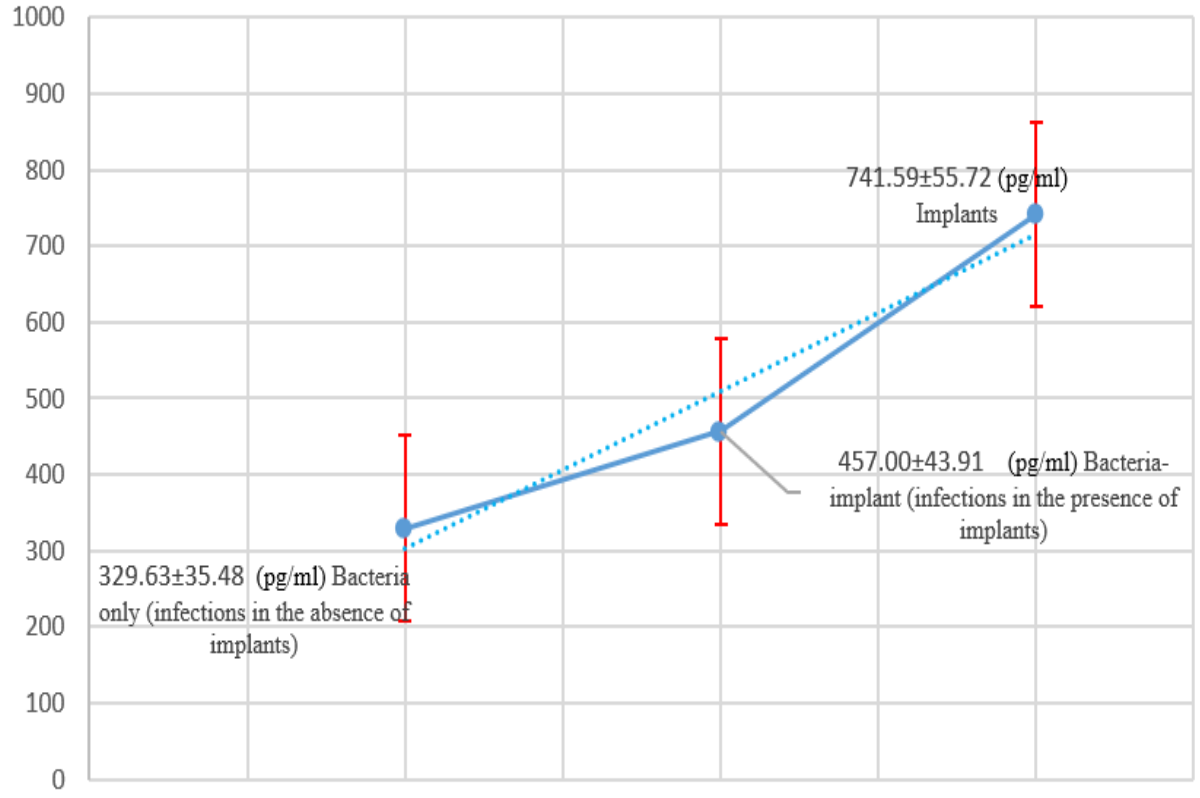
Compounds	Molecular formula	Molecular weight	Compounds	Molecular formula	Molecular weight
2-Heptanone, 6-methyl-	C15H24O	220.35 g/mol	2-Pentene	C6H12	84.16 g/mol
2-Amino-2-methyl-1-propanol	C4H11NO	9.14 g/mol	3-Methylbutanal	C5H10O	86.13 g/mol
Benzaldehyde, 3-methyl	C19H14F6N2O2	416.3 g/mol	3-Methylbutyl acetate	C8H16O3	160.21 g/mol
1-Propanol, 3-amino	C5H13NO	103.16 g/mol	Methacrolein	C4H6O	70.09 g/mol
2-Butanol	C4H10O	74.12 g/mol	2-ethyl-5-methylpyrazine	C7H10N2	122.17 g/mol
Mercaptoacetone	C3H6OS	90.15 g/mol	2-phenylfuran	C10H8O	144.17 g/mol



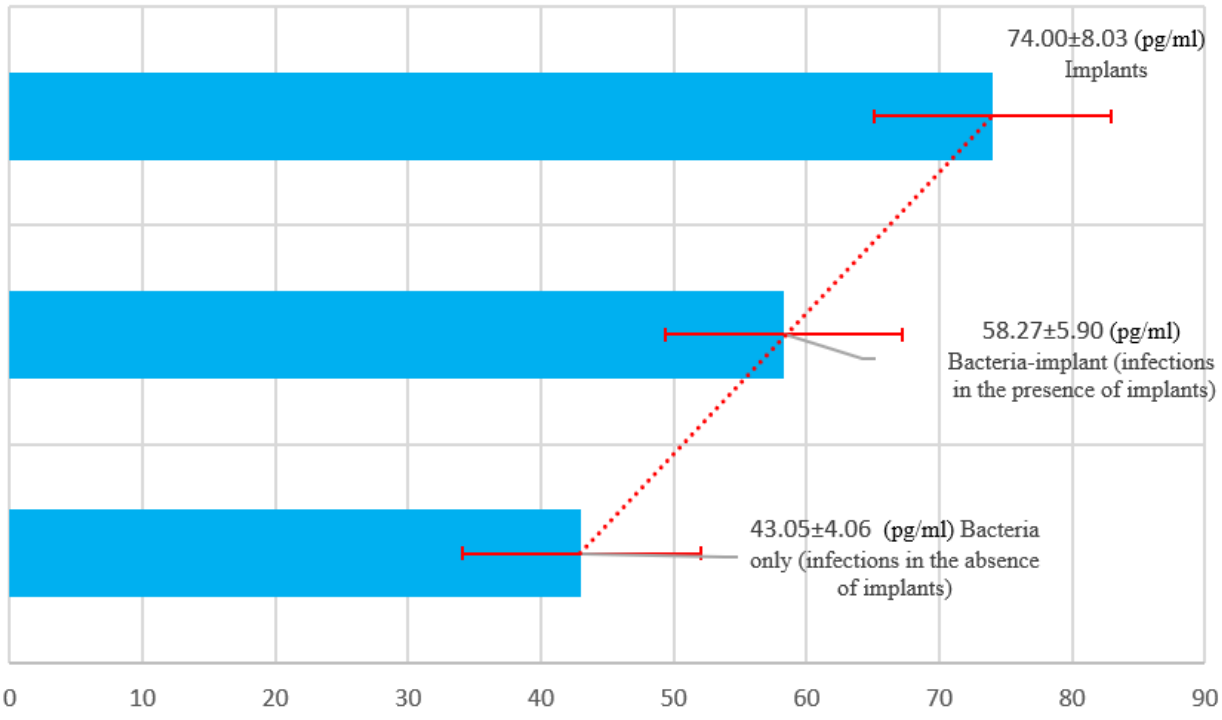
Figurer 1.Sserum level of interleukin IL-6 in mice (Infections in the presence and absence of implants).



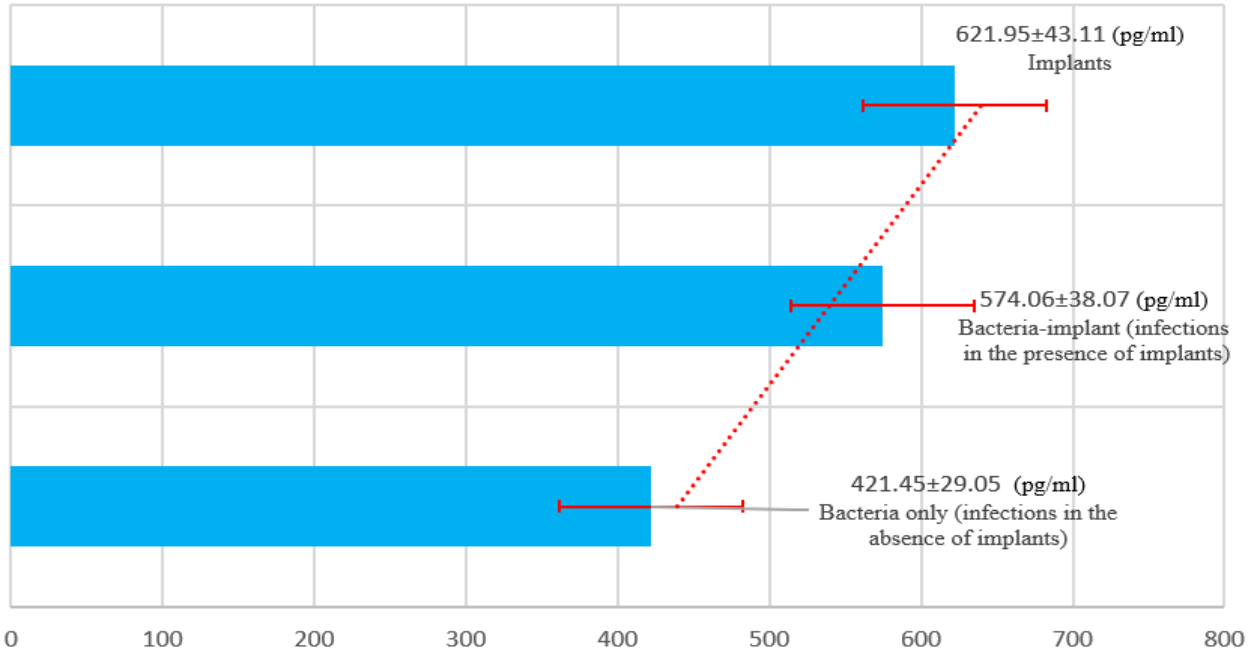
Figurer 2.Sserum level of interleukin IL-18 in mice (Infections in the presence and absence of implants).



Figurer 3.Sserum level of interleukin IL-23 in mice (Infections in the presence and absence of implants).



Figurer 4. Expression of CCL-5 in mice (Infections in the presence and absence of implants).



Figurer 5. Expression of CCL-11 in mice (Infections in the presence and absence of implants).

Among inflammatory cytokines that were detected in the serum of mice by multiplex test were three interleukins as well as two chemokines. Expression of interleukins IL-6, IL-18 and IL-23 serum concentration (pg/ml) Implant, Bacteria-implant (infections in the presence of implants) and Bacteria only (infections in the absence of implants) recorded [32.00±4.09, 22.18±3.81 and 16.45±2.96 (pg/ml)], [250.68±31.04, 125.00±21.29 and 93.85±18.71 (pg/ml)], [741.59±55.72, 457.00±43.91 and 329.63±35.48 (pg/ml)] respectively. The serum levels of CCL-5 and CCL-11 pg/ml recorded [74.00±8.03, 58.27±5.90 and 43.05±4.06 (pg/ml)], [621.95±43.11, 574.06±38.07 and 421.45±29.05 (pg/ml)] respectively. A few volatile components, however, have been described in previous publications. For instance, it has been found that 3-hydroxy-2-butanone is connected with glycolysis [9, 10], while 3-methyl-1-butanol is associated with *Klebsiella pneumoniae*'s catabolism of leucine via the Ehrlich pathway. Furthermore, the uptake of benzaldehyde has been correlated with bacterial growth energy provision. As a rule, throughout development and metabolism, *Klebsiella pneumoniae* is able to produce a number of volatile metabolites. Justification for producing different types of VOCs due to results of a previous systematic analysis on pathogenic bacteria and their VOCs products: There is a close relationship between anaerobic metabolism and the production of organic acids; The fatty acids are oxidised to form alcohols; Eesters are produced through the chemical reaction between acids and alcohols; Ketones are produced by decarboxylation of fatty acid derivatives. However, sources of the other chemicals have not yet been identified [10-13]. Take that into account as these theories should be further studied and tested. Since the GC-MS technique is primarily used for analysing food flavours, no characterised VOCs from medicines are included in the GC-MS library. For this reason several of the volatile metabolites are poorly described. However, it is expected that the database would be expanded as a result of increasing popularity of the GC-MS. Moreover, this work was able to successfully determine the retention indices of these unknown volatile compounds using GC-MS, which indicates future technical development could be used to identify these VOCs that are not yet known.

This also offers a plausible explanation as to why there were no differences in the volatile organic compounds (VOCs) associated with K. pneumoniae detected in this investigation. Thus, it

is important to identify and rapidly characterize CRKP in patients to optimise antibiotic treatment. After the volatolomics of *Klebsiella pneumoniae* were determined, the value of VOCs linked with the bacterium in identifying CRKPs was assessed. As elaborated by previous studies, we decided to add IPM to our investigation, as it can provide insight into the changes in the volatile metabolomics of *Klebsiella pneumoniae*. Through literature, we chose the concentration of 0.25 mg/mL as one of our experimental concentrations for IPM. Interested in studying the volatile IPM products as there is a suggestion by CLSI to use MALDI-TOF to detect Enterobacteriaceae bacteria that produce carbapenemase, including the hydrolysis products of IPM [16–20]. A significant change was observed in the concentration of some volatile organic compounds (VOCs) when both the carbapenemase-positive and negative bacteria were treated with IPM. Interestingly, just three VOC compounds have been detected in the standard strains. In comparison with clinical strains that produce carbapenemase, however, there were over 20 different VOCs in the CSKP [21, 22]. Most significantly, these differential VOCs were mostly associated with *Klebsiella pneumoniae*. Standard strains perform consistently better than clinical strains, perhaps for the following reasons: They are not easily mutated, they are kept under controlled conditions and they are stable. On the other hand, clinical strains are more apt to suffer from mutations due to antibiotic treatment in a healthcare setting. Due to these differences, there may be differences in metabolic processes between clinical and standard strains of Klebsiella pneumoniae. A genomic study of the structure of the *Klebsiella pneumoniae* genome showed that there is a high degree of genetic variation, contributing to the metabolic variation. Furthermore, many studies have demonstrated the possibility of characterising in vitro-cultured bacterial monocultures via volatile organic compound metabolism. Previous studies have shown that, in susceptible bacteria, the consumption of food has increased and the level of metabolites decreased since antibiotics were introduced to the bacterial population [23, 24]. This phenomenon could be the reason for the observed differences in VOCs' metabolic profile in this study. In the present study, GC-IMS was not able to detect imipenem-related VOC molecules, in contrast to previous studies. This may be because of the differences in detection methods or IPM has a short incubation period, so future research should be carried out carefully and in-depth studies should be conducted into this. Alterations in the metabolic profile of VOCs produced by the bacterium led to the ability to differentiate carbapenemase-negative from carbapenemase-positive *Klebsiella pneumoniae* strains [25]. Further studies are needed in the area of the concentration of imipenem in the context of carbapenemase-negative CRKP. When it comes to identifying carbapenemase-positive CRKP strains, the methods used in this investigation seem to be the best. There is a pressing need to identify and classify carbapenemase-positive CRKP strains as soon as possible since different carbapenemase-producing strains have varying antibiotic susceptibilities. This has ramifications for this. Thus, the optimal antibiotic treatment in the clinic is based on timely and accurate identification of carbapenemase-producing CRKP. We then performed a follow up study based on the work done with CLSI approved EDTA-carbapenem inactivation method (eCIM) [26, 27].

There is strong evidence that avibactam inhibits the action of class Class B carbapenemase enzymes are not inhibited by this compound, while it does inhibit class A carbapenemase hydrolase enzymes. Being a new β-lactamase inhibitor that may reversibly covalently bind to enzymes, it belongs to the class of diazabicyclooctanone compounds and has a wide range of antienzyme spectrum. The process by which avibactam works is that the β-lactamase forms a covalent compound with avibactam by attacking its amide bond with a serine nucleophilic assault [28-31]. This combination enables the efficient production of the lactam ring with release of avibactam without hydrolysis of the enzyme. The nucleophilic attack is more effective to increase the rate of ring opening than

cyclisation, significantly lowering the activity of the β-lactamase. The enzyme inhibiting property of Avibactam is also a regenerative one that provides sustained enzyme inhibition. Metal ion chelating agents (such as EDTA) bind to the divalent zinc ions that are required to catalyse reactions in metalloenzymes, and so reduce their activity. The screening of strains that generate metalloenzymes typically makes use of this feature of EDTA. In this study, the metabolic profile of the VOCs of CRKP strains with various carbapenemases was confirmed [32]. The results demonstrated the potential of GC-IMS as a valuable tool to detect the production of CRKPs with class A and B carbapenemases as early as possible. Lastly, a straightforward approach was devised using changes in the relative content of 3-methyl-1-butanol to effectively differentiate carbapenemase-positive strains from non-carbapenemase-positive strains, based on the results reported above. In addition, as reported from our previous studies, these strains underwent significant changes in terms of the 3-methyl-1-butanol content when a carbapenemase inhibitor was introduced.

Advantages of this approach (GC-IMS): *Klebsiella pneumoniae* can be identified from its unique volatile organic compound fingerprint; Differences in the concentration of 3-methyl-1-butanol between groups can be displayed graphically in the GC-IMS spectra; Finally, the data can be compared and analysed by using the relative peak volume of 3-methyl-1-butanol which is semi-quantitative data. Lastly, the test for rapid detection is met as the GC-MS test is performed in only 13 minutes after preparation. K. pneumoniae strains are still needed to be isolated and purified in a clinical setting for this procedure to apply to patient samples in an experiment [33]. This is to make sure that no other bacteria affect the results. It did not explore effects of various different carbapenem antibiotics, effects of various different IPM concentrations or changes in volatile metabolomics over time between various treatments in order to find the best experimental conditions. Subsequent trials and prospective research involving several centers should delve further into these topics. Fifthly, since the number of strains was too low for definite conclusions, we did not investigate the influence of genetic variations of the *Klebsiella pneumoniae* on volatile metabolomics and did not explain the effect of the strains containing different types of carbapenemase on volatile metabolomics. The present study reported fewer volatile compounds in TSB related to *Klebsiella pneumoniae* as compared to similar studies. In previous studies, an attempt had been made to analyse the volatile metabolomics of K. pneumoniae by using GC-MS but the present study was able to identify and report on the presence of carbapenemase generating CRKPs, expanding and enriching the earlier work.

Microorganisms that are cultured from human tissue is the gold standard in identifying the pathogen responsible for nosocomial infections. This approach does have some disadvantages, such as the requirement for well-trained staff to carry it out, the need for invasive sampling of humans and the fact that the results can take up to 3 days to become available, during which antibiotics with a wide spectrum of activity are used empirically. However, it does offer information on antimicrobial resistance. In light of this, researchers are always looking for new ways to combine traditional microbiological methods with analytical data in order to better diagnose bacterial diseases. In the last ten years, numerous important clinical trials have shown that breath analysis can be helpful in detecting bacterial presence in the respiratory tract or bloodstream of severely sick patients. This method is based on the ability to quantify and qualify the volatile metabolites of bacteria in exhaled breath gas of patients with suspected infections, with the use of mass spectrometric methods. Some bacterial volatile organic compounds (VOCs) are present in the breath of patients with ventilator-associated pneumonia (VAP) and another solid study found that the levels of these metabolites change in the course of pneumonia and seem to be a measure of the level of infection. Another clinical trial showed that breath testing can be used to differentiate bacterial colonization of MV patients

from the actual cause of VAP. It may also help to determine if the causative organism (Acinetobacter baumannii) is present in the patient's lower respiratory tract. However, because breath analysis is non-invasive, despite these proofs, no volatile biomarker has yet been clinically significant for the reliable prediction of the development of bacterial infections or sepsis. The analytical approach and strategy to interpreting the data is not uniform and study designs often involve recruiting a limited sample of patients from a single center. As a whole, this can give rise to false conclusions and a random clustering of variables studied. It is essential to be able to accurately identify the metabolite of interest for data interpretation. This includes validation in a model in vitro experiment using microorganisms that it is of bacterial origin. Use of reference standards for identification of VOCs, including a preliminary match to the MS library, is recommended. This approach provides a better understanding of the clinical outcome of the study results by identifying a metabolic pathway that supports the production of VOCs by bacteria. To study possible mechanisms of volatile organic compound (VOC) emission and/or uptake, and to elucidate the metabolites causing antimicrobial resistance, this study used in vitro experiments as models and employed *Klebsiella pneumonia*, which is known to show both carbapenem susceptibility and resistance, as references. This was followed by a study on effects of supplementing bacterial culture with antibiotics on the VOC production [34]. The answer is to compare the Klebsiella pneumoniae (KP) volatile organic compound (VOC) profiles from clinical strains with VAP patients to the profiles of the KP reference strains.

5-Conclusions

This is the first work that we are aware of that uses GC-MS to thoroughly examine the volatile metabolomic of *Klebsiella pneumoniae* in tissue culture broth. This study is an additional example of the utility of GC-MS for identifying the volatile metabolomic of *Klebsiella pneumoniae* to validate the value of this technology for identification of carbapenemase-producing CRKPs. However, to corroborate our findings, we need to optimize our experimental conditions and collect a bigger array, thus the results of this exploratory investigation are preliminary. In this work, the kinetics of VOC release from the untreated and imipenem treated *Klebsiella pneumoniae* are explained. VOC metabolism was seen to have three different responses; proportional to the number of bacteria, release with a transient maximum during maximum growth activity, and absorption as a material or energy source. The unexpected carbon methyl ketones (2-pentanone, 2-heptanone, and 2-nonanone) were used as indicators for antimicrobial resistance since these compounds were released significantly higher by the resistant *Klebsiella pneumoniae* strain than the susceptible strain. The treated susceptible strain culture was found to have a rapid decline in the levels of several volatile metabolites along with the elimination of bacteria. It is possible to determine a target microbe by measuring certain volatiles in the headspace of the culture. This is promising for medical microbiology labs as a diagnostic tool as it might soon be available. A major aim would be to develop a non-invasive diagnostic tool for bacterial disease, in the field, using breath analysis, based on these data. Further model in vitro studies with bacterial cultures are needed to pave the way for clinical trials in multiple centers that will establish the clinical relevance of selected volatile biomarkers. To reach such a high target as non-invasive diagnosis of pneumonia with the differentiation of the causative agent, it is necessary to assign a specific set of metabolites to the specific bacteria and not just one compound. This panel of metabolites will then have to be confirmed in multi-center clinical studies which include well-controlled cohorts of MV patients. Our in vitro research has provided the basis for this strategy, revealing that the carbapenem-resistant and susceptible *Klebsiella pneumoniae* synthesize volatile organic compounds (VOCs) under both normal and stressed conditions, with similarities and differences. Further in vitro studies are needed with clinically relevant microorganisms to identify the most promising metabolites for application in a non-invasive,

point-of-care diagnostic of bacterial infection through breath gas analysis.

6-Conflict of interest

The author declare that theyhave no conflict of interest

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