

Dose-Dependent Interleukin-6 Responses Induced by Clinical Bacterial Isolates in an Ex Vivo Whole-Blood Model: A Laboratory-Based Experimental Study

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Abstract

Background: Interleukin-6 (IL-6) is a key pro-inflammatory cytokine involved in the early immune response to bacterial infections. The magnitude of IL-6 production is influenced by multiple factors, including bacterial characteristics, microbial burden, and host immune responsiveness.

Although cytokine responses to bacterial pathogens have been widely investigated, limited information is available on how increasing bacterial inoculum affects IL-6 production following stimulation with clinically relevant bacterial isolates obtained from routine patient specimens. Understanding these dose-dependent responses may improve characterization of early host–pathogen interactions and provide baseline information for future investigations of inflammatory biomarker regulation during bacterial infection.

Results: Clinical bacterial isolates demonstrated heterogeneous IL-6 response profiles across the tested inoculum concentrations. Under the experimental conditions seven of thirteen isolates showed progressive increases in IL-6 concentration across the three bacterial concentrations, while six demonstrated variable trajectories. When the lowest and highest inocula were compared, twelve isolates showed equal or higher IL-6 concentrations at 1×10^8 CFU/mL. The largest fold increases were observed for *Citrobacter freundii* (3.29-fold), *Proteus vulgaris* (2.24-fold), and *Pseudomonas aeruginosa* (2.20-fold), whereas *Proteus mirabilis* showed minimal overall change (0.99-fold).

Conclusion: Exposure of human whole blood to clinical bacterial isolates resulted in variable but generally dose-dependent IL-6 responses in an ex vivo stimulation model.. Increasing bacterial concentration was associated with enhanced cytokine production, although the magnitude of response differed among bacterial species. These findings provide descriptive evidence that bacterial burden influences early inflammatory responses and support the application of ex vivo whole blood stimulation models for investigating host-pathogen interactions.

BACKGROUND

Bacterial infections remain a major challenge to global health, not only because of pathogen-associated disease burden but also because of the complex interactions between invading microorganisms and host immune responses.. The outcome of bacterial infection depends not only on the virulence characteristics of the invading pathogen but also on the magnitude and regulation of the host immune response. Early recognition of invading microorganisms by the innate immune system initiates a cascade of inflammatory events that are essential for pathogen clearance but, when dysregulated, may contribute to tissue injury and disease progression (1, 2).

Among the cytokines involved in early host defense, IL-6 is one of the most rapidly induced and biologically versatile mediators of inflammation. IL-6 is produced predominantly by monocytes, macrophages, dendritic cells, endothelial cells, fibroblasts, and other immune cells following stimulation by bacterial pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS),

peptidoglycan, lipoteichoic acid, flagellin, and bacterial lipoproteins. Through activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway, IL-6 regulates acute-phase protein synthesis, neutrophil recruitment, B-cell differentiation, T-cell activation, and communication between innate and adaptive immunity. Consequently, IL-6 has become an important biomarker of inflammation, infection severity, and host immune activation in both clinical practice and experimental research (2, 3).

The intensity of IL-6 production is influenced by multiple factors, including bacterial species, virulence determinants, host immune status, and bacterial burden. Increasing bacterial inoculum exposes immune cells to larger quantities of microbial ligands capable of activating pattern recognition receptors, particularly Toll-like receptors (TLRs), thereby amplifying intracellular inflammatory signaling. Recognition of bacterial components by innate immune receptors activates intracellular signaling pathways, including nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways, resulting in the transcription and release of pro-inflammatory mediators such as IL-6. Experimental evidence indicates that increasing bacterial load enhances cytokine secretion during the early stages of infection, suggesting that bacterial concentration represents one factor that can influence the magnitude of innate immune activation (2, 4, 5). Characterizing these dose-dependent inflammatory responses may improve our understanding of host-pathogen interactions and provide insights into the biological mechanisms underlying variable inflammatory responses observed during bacterial infections.

Ex vivo whole blood stimulation models provide a physiologically relevant platform for investigating innate immune responses because they preserve the natural cellular composition of peripheral blood while maintaining interactions among leukocytes, plasma proteins, complement components, and soluble inflammatory mediators. Unlike isolated peripheral blood mononuclear cell cultures, whole blood assays require minimal sample manipulation and more closely reflect the in vivo immune environment. These characteristics have made whole blood stimulation models increasingly valuable for evaluating cytokine responses following exposure to bacterial pathogens and microbial products (4, 6).

Most previous investigations evaluating IL-6 responses have used reference laboratory strains, purified bacterial components such as lipopolysaccharide, or experimentally prepared microorganisms. Comparatively few studies have examined dose-dependent cytokine responses induced by clinically derived bacterial isolates recovered directly from infected patients. Clinical isolates exhibit considerable heterogeneity in their genetic background, virulence characteristics, and host adaptation, factors that may influence their capacity to stimulate inflammatory responses independently of antimicrobial susceptibility profiles. Describing the inflammatory responses induced by clinically relevant pathogens therefore provides information that may better reflect host-pathogen interactions encountered in routine clinical settings.

Furthermore, although numerous studies have investigated cytokine responses during bacterial infections, relatively limited information exists regarding how increasing concentrations of diverse

clinical bacterial isolates influence IL-6 production under standardized ex vivo conditions. Establishing these descriptive response patterns may provide baseline data for future studies investigating bacterial pathogenicity, immune activation, and inflammatory biomarker kinetics. To our knowledge, few studies have systematically evaluated dose-dependent IL-6 responses induced by multiple clinically derived bacterial isolates using a standardized ex vivo whole-blood stimulation model.

Therefore, the present study aimed to evaluate dose-dependent IL-6 responses following ex vivo stimulation of human whole blood with increasing concentrations of clinical bacterial isolates recovered from bloodstream and urinary tract infections. By characterizing changes in IL-6 production across standardized bacterial inocula, this study seeks to improve understanding of early inflammatory responses induced by clinically important bacterial pathogens.

METHODS

Study Aim

To characterize dose-dependent IL-6 responses induced by clinical bacterial isolates in an ex vivo whole-blood model and describe variations in cytokine production across different bacterial inoculum concentrations.

Study Design

An exploratory descriptive laboratory-based experimental study was conducted using an **ex vivo whole blood stimulation model** to characterize dose-dependent IL-6 responses induced by clinical bacterial isolates. The study evaluated changes in IL-6 production following stimulation with increasing bacterial inoculum concentrations under standardized laboratory conditions.

Study Setting

This laboratory-based experimental study was conducted at the Kilimanjaro Clinical Research Institute (KCRI) and the Center of Excellence Clinical Laboratory of Kilimanjaro Christian Medical Centre (KCMC) Referral Hospital, Moshi, Tanzania. KCMC is a tertiary-level referral and teaching hospital serving the northern regions of Tanzania and functions as the clinical training facility for KCMC University. The hospital provides specialized healthcare services to a diverse patient population and receives clinical specimens from a large catchment area, generating a valuable repository of clinically characterized bacterial isolates for biomedical research.

The KCMC Clinical Microbiology Laboratory routinely performs bacterial identification from clinical specimens, including blood and urine samples, and maintains archived collections of clinically significant bacterial isolates. These archived isolates provided the bacterial stimuli used in the present study to investigate host inflammatory responses.

Laboratory procedures, including retrieval and preparation of archived bacterial isolates, standardization of bacterial inocula, ex vivo whole-blood stimulation assays, and quantification of IL-6 using enzyme-linked immunosorbent assay (ELISA), were performed at KCRI and the Center of Excellence Clinical Laboratory under standardized biosafety and quality assurance procedures. The availability of clinical microbiology infrastructure and immunological laboratory capacity at these facilities provided an appropriate setting for evaluating dose-dependent cytokine responses induced by clinically relevant bacterial pathogens.

Eligibility Criteria

This study included bacterial isolates recovered from blood and urine specimens of patients who received care at KCMC, Moshi, Tanzania. Only clinically significant bacterial isolates documented in the Clinical Microbiology Laboratory records and archived in the KCMC biorepository were considered for inclusion.

To be eligible, each bacterial isolate was required to have complete laboratory documentation, including specimen source, bacterial species identification, and corresponding microbiological records. Only viable archived isolates that could be successfully retrieved, subcultured, and standardized to the predetermined bacterial inoculum concentrations (1×10^6 , 1×10^7 , and 1×10^8) Colony Forming Unit (CFU)/mL were included in the ex vivo whole blood stimulation experiments. Both commonly isolated pathogens and less frequently encountered bacterial species were eligible for inclusion, provided they fulfilled all laboratory quality requirements and were available for cytokine stimulation assays.

Healthy blood donors were eligible to participate if they were adults (≥ 18 years), were apparently healthy at the time of blood collection, had no clinical evidence of acute infection or chronic inflammatory disease, and provided written informed consent before participation. Donors receiving immunosuppressive therapy or presenting with any condition likely to alter normal immune function were excluded from participation.

Bacterial isolates were excluded if they were non-viable following retrieval from the biorepository, failed to produce adequate growth during subculture, demonstrated contamination or mixed microbial growth, or could not be standardized to the required bacterial inoculum concentrations. Isolates with incomplete laboratory documentation or those failing laboratory quality control procedures were also excluded from the study.

These eligibility criteria were established to ensure the use of well-characterized clinical bacterial isolates, maintain laboratory quality standards, and provide reliable assessment of dose-dependent IL-6 responses in the ex vivo whole blood stimulation model.

Source of Bacterial Isolates

Archived clinical bacterial isolates recovered from routine diagnostic blood and urine specimens were obtained from the KCMC Clinical Microbiology Laboratory. The collection included clinically significant

Gram-positive and Gram-negative bacterial species commonly associated with bloodstream and urinary tract infections. Isolates were selected to represent a range of clinically important pathogens encountered during routine diagnostic practice.

Sample Size

This was an exploratory laboratory-based study employing an ex vivo whole blood stimulation model to evaluate dose-dependent IL-6 responses induced by clinical bacterial isolates. Given the exploratory nature of the study, the limited availability of archived clinical isolates, and the absence of published data to support formal power calculations for this experimental model, a formal sample size estimation was not performed.

Eligible bacterial isolates were selected from the archived collection of the KCMC Clinical Microbiology Laboratory based on the predefined eligibility criteria. The final number of isolates included in the study was determined by the availability of viable, well-characterized archived isolates suitable for standardized laboratory experimentation.

For immune stimulation experiments, whole blood was collected from **two apparently healthy adult donors** who satisfied the predefined eligibility criteria. Each bacterial isolate was prepared at three standardized inoculum concentrations (1×10^6 , 1×10^7 , and 1×10^8 CFU/mL) and independently used to stimulate whole blood from both donors under identical experimental conditions. This design generated replicate cytokine measurements across bacterial concentrations and donors, enabling descriptive evaluation of dose-dependent IL-6 response patterns.

As the primary objective of this study was to characterize cytokine response trends rather than to test predefined hypotheses, descriptive analyses were considered appropriate for the available experimental data. Accordingly, the study was designed to generate preliminary descriptive data rather than to test predefined statistical hypotheses or estimate population-level effects.

Healthy Blood Donors

Due to the exploratory nature of the study, donor samples were used to generate preliminary descriptive response patterns. Whole blood used for the stimulation experiments was obtained from **two apparently healthy adult volunteers** recruited for the study. Donors were selected because they had no clinical evidence of acute infection, chronic inflammatory disease, autoimmune disorders, or immunodeficiency at the time of blood collection. Individuals receiving immunosuppressive medication or presenting with symptoms suggestive of active infection were excluded.

Peripheral venous blood was collected aseptically into lithium heparinized tubes and processed immediately for ex vivo stimulation experiments. Each donor's whole blood was stimulated independently with every bacterial isolate at the three standardized bacterial inoculum concentrations, allowing the consistency of observed IL-6 response patterns to be examined across the two donors.

Preparation of Bacterial Inocula

Archived isolates were subculture on appropriate culture media and incubated under recommended microbiological conditions to obtain pure colonies. Individual colonies were suspended in sterile phosphate-buffered saline (PBS), and bacterial density was standardized using the 0.5 McFarland turbidity standard. The suspensions were subsequently adjusted to obtain final bacterial concentrations of 1×10^6 , 1×10^7 , and 1×10^8 (CFU)/mL.

To ensure laboratory safety while preserving bacterial structural components responsible for immune stimulation, bacterial suspensions were heat-inactivated before whole blood stimulation. Heat inactivation eliminated bacterial viability while retaining PAMPs capable of activating innate immune receptors and inducing cytokine production. Successful inactivation was confirmed by sub culturing representative aliquots on blood agar plates to verify the absence of bacterial growth.

Whole Blood Collection

Peripheral venous blood was collected from healthy adult volunteers with no clinical evidence of acute infection or chronic inflammatory disease. Blood samples were collected aseptically into sterile lithium heparin tubes to preserve leukocyte viability and functional immune responses during ex vivo stimulation. All blood samples were processed promptly following collection to minimize pre-analytical variation.

Ex Vivo Whole Blood Stimulation

Whole blood aliquots were dispensed into sterile culture tubes before stimulation with heat-inactivated bacterial suspensions at the three standardized inoculum concentrations (1×10^6 , 1×10^7 , and 1×10^8 CFU/mL). Unstimulated whole blood served as the negative control.

Following stimulation, samples were incubated at **37°C in a humidified atmosphere containing 5% CO₂ for 48 hours** to allow cytokine production. At the end of incubation, cultures were centrifuged, and the supernatants were carefully harvested and stored at **-80°C** until cytokine analysis.

Quantification of Interleukin-6

IL-6 concentrations in the collected whole blood culture supernatants were quantified using a commercially available sandwich ELISA according to the manufacturer's instructions. Standards, controls, and study samples were included in each assay run, and samples were analyzed in accordance with the assay protocol. IL-6 was selected as the primary outcome measure because it is an early pro-inflammatory cytokine that is rapidly induced following bacterial recognition and is widely used as a biomarker of innate immune activation.

Following completion of the ELISA procedure, optical density (OD) was measured using a calibrated BioTek ELx808 microplate reader at the wavelength specified by the assay manufacturer. The OD

readings were used to generate a standard calibration curve, from which the IL-6 concentration of each study sample was determined. Final IL-6 concentrations were expressed in picograms per millilitre (pg/mL).

The resulting IL-6 concentrations, rather than the raw OD values, were used for subsequent descriptive analysis of cytokine responses across the three bacterial inoculum concentrations.

Data Management and Analysis

Laboratory results were entered into Microsoft Excel and verified for completeness and consistency before analysis. Descriptive analyses were performed using Microsoft Excel and IBM SPSS Statistics version 25.

IL-6 concentrations were summarized for each bacterial isolate at the three inoculum concentrations (1×10^6 , 1×10^7 , and 1×10^8 CFU/mL). Dose-dependent response patterns were described using tables, line graphs, and heat maps.

To characterize the magnitude of cytokine induction, the following descriptive measures were calculated for each bacterial isolate:

- Absolute IL-6 concentration at each bacterial inoculum.
- Fold change in IL-6 production between 1×10^6 and 1×10^8 CFU/mL.
- Percentage increase in IL-6 production from the lowest to the highest bacterial concentration.
- Maximum observed IL-6 response.
- Overall response trajectory (increasing, plateauing, or variable).

Given the exploratory nature of the study and the limited number of bacterial isolates, inferential statistical analyses were not performed. The analysis focused on describing observed dose-response patterns rather than testing statistical significance.

Results

Characteristics of Clinical Bacterial Isolates

A total of 13 clinically significant bacterial isolates recovered from routine diagnostic blood and urine specimens at KCMC were included in this study. The selected isolates represented bacterial pathogens commonly associated with bloodstream and urinary tract infections and comprised both Gram-positive and Gram-negative species. Archived isolates were retrieved from the KCMC Clinical Microbiology biorepository, reactivated through subculture, confirmed for viability and purity, and standardized to defined bacterial concentrations for subsequent ex vivo whole-blood stimulation experiments.

The bacterial collection comprised isolates recovered from blood and urine specimens, representing pathogens associated with two clinically important infection syndromes encountered in routine hospital

practice. Each isolate was standardized to three predefined bacterial inoculum concentrations (1×10^6 , 1×10^7 , and 1×10^8 CFU/mL) before stimulation of whole blood obtained from two apparently healthy adult donors.

Overall, 39 bacterial stimulation conditions (13 isolates $\times$ 3 inoculum concentrations) were evaluated for each donor, generating paired IL-6 cytokine response measurements across different bacterial exposure levels. These data were used to descriptively evaluate isolate-specific and dose-dependent patterns of IL-6 production in the ex vivo whole-blood model.

Table 1: Characteristics of Clinical Bacterial Isolates Included in the Ex Vivo Whole-Blood Stimulation Experiments

Bacterial Species	Specimen Source	Gram Reaction	Number of Isolates
<i>Acinetobacter baumannii</i>	Blood	Gram-negative	1
<i>Citrobacter freundii</i>	Blood	Gram-negative	1
<i>Escherichia coli</i>	Blood	Gram-negative	1
<i>Klebsiella pneumoniae</i>	Blood	Gram-negative	1
<i>Proteus mirabilis</i>	Blood	Gram-negative	1
<i>Pseudomonas aeruginosa</i>	Blood	Gram-negative	1
<i>Staphylococcus aureus</i>	Blood	Gram-positive	1
<i>Citrobacter koseri</i>	Urine	Gram-negative	1
<i>Enterococcus</i> spp.	Urine	Gram-positive	1
<i>Klebsiella oxytoca</i>	Urine	Gram-negative	1
<i>Morganella morganii</i>	Urine	Gram-negative	1
<i>Proteus vulgaris</i>	Urine	Gram-negative	1
<i>Serratia marcescens</i>	Urine	Gram-negative	1

Note: Each bacterial species was represented by one archived clinical isolate recovered from routine diagnostic blood or urine specimens at KCMC Center of Excellence Clinical Laboratory. Gram classification was based on routine microbiological identification procedures.

Dose-Dependent IL-6 Responses across Clinical Bacterial Isolates

Exposure of whole blood to clinical bacterial isolates resulted in variable but generally dose-dependent IL-6 responses across the three bacterial inoculum concentrations. Overall, increasing the bacterial concentration from 1×10^6 to 1×10^8 CFU/mL was associated with higher IL-6 concentrations in most isolates, although the magnitude and trajectory of the response differed among bacterial species.

The most pronounced increase in IL-6 concentration was observed for *Citrobacter freundii*, for which IL-6 increased from 6,190.35 pg/mL at 1×10^6 CFU/mL to 9,414.34 pg/mL at 1×10^7 CFU/mL and further to 20,363.74 pg/mL at 1×10^8 CFU/mL. This represented a 3.29-fold increase between the lowest and highest bacterial concentrations, corresponding to a 229.0% increase. A similarly marked increase was observed for *Proteus vulgaris*, with IL-6 increasing from 7,711.10 pg/mL at 1×10^6 CFU/mL to 7,954.42 pg/mL at 1×10^7 CFU/mL and 17,261.41 pg/mL at 1×10^8 CFU/mL, corresponding to a 2.24-fold increase and a 123.9% increase between the lowest and highest inocula.

Pseudomonas aeruginosa also demonstrated a pronounced dose-associated increase. IL-6 increased from 10,509.28 pg/mL at 1×10^6 CFU/mL to 20,607.06 pg/mL at 1×10^7 CFU/mL and 23,101.09 pg/mL at 1×10^8 CFU/mL. The corresponding fold change between 1×10^6 and 1×10^8 CFU/mL was 2.20, representing a 119.8% increase.

Moderate increases were observed among several other isolates. *Klebsiella oxytoca* increased from 19,451.29 pg/mL at 1×10^6 CFU/mL to 24,196.03 pg/mL at 1×10^8 CFU/mL, representing a 1.24-fold increase (24.4%). *Enterococcus* spp. increased from 16,896.43 pg/mL to 20,546.23 pg/mL, corresponding to a 1.22-fold increase (21.6%), while *Acinetobacter baumannii* increased from 17,565.56 pg/mL to 21,154.53 pg/mL, corresponding to a 1.20-fold increase (20.4%). *Morganella morganii* demonstrated a similar pattern, increasing from 16,166.47 pg/mL to 19,268.80 pg/mL, corresponding to a 1.19-fold increase (19.2%).

Smaller increases between the lowest and highest inocula were observed for *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Citrobacter koseri*, and *Serratia marcescens*. *Escherichia coli* increased from 20,242.08 pg/mL at 1×10^6 CFU/mL to 22,796.94 pg/mL at 1×10^8 CFU/mL, corresponding to a 1.13-fold increase (12.6%). *Klebsiella pneumoniae* increased from 19,390.46 pg/mL to 21,397.85 pg/mL, representing a 1.10-fold increase (10.4%). The corresponding increases for *Staphylococcus aureus*, *Citrobacter koseri*, and *Serratia marcescens* were 6.9%, 10.5%, and 3.2%, respectively.

Not all isolates demonstrated a progressive increase across the three concentrations. *Acinetobacter baumannii*, *Escherichia coli*, *Proteus mirabilis*, *Staphylococcus aureus*, *Citrobacter koseri*, and *Serratia marcescens* showed variations in IL-6 concentration at the intermediate inoculum of 1×10^7 CFU/mL. For example, IL-6 production by *Citrobacter koseri* decreased from 19,694.61 pg/mL at 1×10^6 CFU/mL to 8,319.40 pg/mL at 1×10^7 CFU/mL before increasing to 21,762.83 pg/mL at 1×10^8 CFU/mL. Similarly, *Proteus mirabilis* showed a slight reduction from 22,736.11 pg/mL at 1×10^6 CFU/mL to 20,789.55 pg/mL at 1×10^7 CFU/mL, followed by an increase to 22,553.62 pg/mL at 1×10^8 CFU/mL. The overall

change between the lowest and highest concentrations was therefore minimal, corresponding to a fold change of 0.99 and a -0.8% change.

Taken together, the descriptive findings demonstrate considerable heterogeneity in IL-6 response patterns among the clinical bacterial isolates. While several isolates exhibited a clear increase in IL-6 production with increasing bacterial concentration, others showed a less consistent response across the three inoculum levels. The largest relative increases were observed for *Citrobacter freundii*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*, whereas *Proteus mirabilis* showed the smallest overall change between the lowest and highest bacterial concentrations.

Summary of Dose-Response Patterns

Seven of thirteen isolates demonstrated progressive increases in IL-6 concentration across the tested inoculum concentrations under the experimental conditions.

These isolates were *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus* spp., *Klebsiella oxytoca*, *Morganella morganii*, and *Proteus vulgaris*. The remaining six isolates showed variable trajectories, characterized by a reduction or fluctuation in IL-6 concentration at the intermediate inoculum.

When only the lowest and highest inoculum concentrations were compared, twelve of the thirteen isolates had an IL-6 concentration at 1×10^8 CFU/mL that was greater than or equal to the concentration observed at 1×10^6 CFU/mL. The largest relative increase was observed for *Citrobacter freundii* (3.29-fold), followed by *Proteus vulgaris* (2.24-fold) and *Pseudomonas aeruginosa* (2.20-fold). In contrast, *Proteus mirabilis* showed a slight overall decrease, with a fold change of 0.99.

The highest absolute IL-6 concentration recorded at 1×10^8 CFU/mL was observed for *Klebsiella oxytoca* (24,196.03 pg/mL), followed by *Pseudomonas aeruginosa* (23,101.09 pg/mL) and *Escherichia coli* (22,796.94 pg/mL). These findings demonstrate heterogeneity in both the magnitude and trajectory of IL-6 responses among the clinical bacterial isolates evaluated.

Fold-Change Analysis of IL-6 Responses

To further characterize the magnitude of the IL-6 response to increasing bacterial inoculum, fold change was calculated for each bacterial isolate by comparing the IL-6 concentration observed at the highest bacterial concentration (1×10^8 CFU/mL) with that observed at the lowest concentration (1×10^6 CFU/mL). The analysis provided a descriptive measure of the change in cytokine response across the experimental concentration range.

The greatest fold increase was observed for *Citrobacter freundii*, with IL-6 increasing from 6,190.35 pg/mL at 1×10^6 CFU/mL to 20,363.74 pg/mL at 1×10^8 CFU/mL, corresponding to a 3.29-fold increase.

This was followed by *Proteus vulgaris*, which demonstrated a 2.24-fold increase, from 7,711.10 pg/mL to 17,261.41 pg/mL. *Pseudomonas aeruginosa* demonstrated a 2.20-fold increase, with IL-6 increasing from 10,509.28 pg/mL to 23,101.09 pg/mL.

Moderate fold increases were observed for *Klebsiella oxytoca* (1.24-fold), *Enterococcus* spp. (1.22-fold), *Acinetobacter baumannii* (1.20-fold), and *Morganella morganii* (1.19-fold). Smaller increases were observed for *Escherichia coli* (1.13-fold), *Citrobacter koseri* (1.11-fold), *Klebsiella pneumoniae* (1.10-fold), *Staphylococcus aureus* (1.07-fold), and *Serratia marcescens* (1.03-fold).

Proteus mirabilis demonstrated the smallest change between the lowest and highest bacterial concentrations, with IL-6 decreasing slightly from 22,736.11 pg/mL at 1×10^6 CFU/mL to 22,553.62 pg/mL at 1×10^8 CFU/mL. The resulting fold change was 0.99, corresponding to a 0.8% decrease.

Overall, twelve of the thirteen isolates demonstrated an IL-6 concentration at 1×10^8 CFU/mL that was equal to or greater than the concentration observed at 1×10^6 CFU/mL. The magnitude of the observed change varied substantially between isolates, ranging from 0.99-fold for *Proteus mirabilis* to 3.29-fold for *Citrobacter freundii*. These observations demonstrate heterogeneity in the magnitude of IL-6 responses to increasing bacterial inoculum among the clinical isolates evaluated.

Table 2: Dose-dependent IL-6 responses and fold changes among clinical bacterial isolates at three bacterial inoculum concentrations

Bacterial Species	IL-6 (10 ⁶)	IL-6 (10 ⁷)	IL-6 (10 ⁸)	Fold Change	% Increase	Response Pattern
<i>Acinetobacter baumannii</i>	17565.56	21397.85	21154.53	1.20	20.4%	Fluctuating
<i>Citrobacter freundii</i>	6190.35	9414.34	20363.74	3.29	229.0%	Increasing
<i>Escherichia coli</i>	20242.08	18295.52	22796.94	1.13	12.6%	Fluctuating
<i>Klebsiella pneumoniae</i>	19390.46	20728.72	21397.85	1.10	10.4%	Increasing
<i>Proteus mirabilis</i>	22736.11	20789.55	22553.62	0.99	-0.8%	Fluctuating
<i>Pseudomonas aeruginosa</i>	10509.28	20607.06	23101.09	2.20	119.8%	Increasing
<i>Staphylococcus aureus</i>	20363.74	19572.95	21762.83	1.07	6.9%	Fluctuating
<i>Citrobacter koseri</i>	19694.61	8319.4	21762.83	1.11	10.5%	Fluctuating
<i>Enterococcus</i> spp.	16896.43	18356.35	20546.23	1.22	21.6%	Increasing
<i>Klebsiella oxytoca</i>	19451.29	20546.23	24196.03	1.24	24.4%	Increasing
<i>Morganella morganii</i>	16166.47	17383.07	19268.8	1.19	19.2%	Increasing
<i>Proteus vulgaris</i>	7711.1	7954.42	17261.41	2.24	123.9%	Increasing
<i>Serratia marcescens</i>	21032.87	19512.12	21702	1.03	3.2%	Fluctuating

Note: IL-6 concentrations are presented as pg/mL. Fold change was calculated as IL-6 concentration at 1×10^8 CFU/mL divided by IL-6 concentration at 1×10^6 CFU/mL. Percentage increase was calculated as $[(\text{IL-6 at } 1 \times 10^8 \text{ CFU/mL} - \text{IL-6 at } 1 \times 10^6 \text{ CFU/mL}) / \text{IL-6 at } 1 \times 10^6 \text{ CFU/mL}] \times 100$. CFU, IL-6.

Discussion

This exploratory study evaluated IL-6 responses induced by thirteen clinical bacterial isolates at three standardized bacterial inoculum concentrations using an ex vivo whole-blood model. Because only one isolate per bacterial species and two blood donors were included, the findings should be interpreted as exploratory observations rather than evidence of species-specific inflammatory properties. The principal finding was that IL-6 responses varied considerably among the isolates and across the tested inoculum concentrations. Seven of the thirteen isolates demonstrated a progressive increase in IL-6 concentration from 1×10^6 to 1×10^8 CFU/mL, while the remaining isolates exhibited variable response trajectories. When the lowest and highest inocula were compared directly, twelve of the thirteen isolates showed equal or higher IL-6 concentrations at 1×10^8 CFU/mL. These findings suggest that increasing bacterial

inoculum was generally associated with increased IL-6 production under the experimental conditions, although the magnitude and consistency of the response differed substantially between isolates.

Dose-associated IL-6 responses

The observed increase in IL-6 concentration with increasing bacterial inoculum is biologically consistent with the activation of innate immune recognition pathways following exposure to bacterial components. Bacterial PAMPs are detected by host pattern-recognition receptors (PRRs), including TLRs, which initiate intracellular signaling cascades that regulate inflammatory and antimicrobial responses. Recent advances in innate immunology have further emphasized the central role of PRRs in translating microbial recognition into coordinated inflammatory responses(7, 8). IL-6 is an important downstream inflammatory mediator with pleiotropic functions, contributing to the transition between innate and adaptive immunity and regulating acute-phase responses during infection and inflammation(9).

The use of graded bacterial concentrations in the study allowed the relationship between the magnitude of the bacterial stimulus and IL-6 production to be examined descriptively. Several isolates demonstrated progressively higher IL-6 concentrations with increasing inoculum, indicating that, under the experimental conditions used, a greater bacterial stimulus was generally accompanied by increased IL-6 production. However, this pattern was not uniform across all isolates, suggesting that the magnitude of the IL-6 response was influenced by both bacterial concentration and isolate-specific characteristics. This observation is consistent with recent evidence demonstrating that ex vivo whole-blood models can capture distinct host-response profiles following exposure to different bacterial strains. In a 2024 study, Chick et al. demonstrated strain-specific differences in multiple host-response parameters, including IL-6, following bacterial stimulation of whole blood, and showed that IL-6 contributed to the differentiation of bacterial genera and species(10). These findings support the utility of ex vivo whole-blood models for characterizing differences in host inflammatory responses to clinically relevant bacterial stimuli.

However, the present findings also demonstrate that the relationship between bacterial inoculum and IL-6 production was not uniformly linear across all isolates. Several isolates showed a reduction in IL-6 concentration at the intermediate inoculum, followed by an increase at 1×10^8 CFU/mL. This variability suggests that bacterial concentration alone may not fully determine the magnitude of the inflammatory response. Differences in bacterial species and strain characteristics may contribute to variation in host-cell recognition and downstream cytokine production. Such differences may arise from variation in bacterial surface components and PAMPs, which can engage different pattern-recognition receptor pathways and influence the magnitude and kinetics of inflammatory signaling. Recent ex vivo whole-blood research has similarly demonstrated distinct IL-6 response profiles following stimulation with different bacterial strains, supporting the importance of bacterial-specific characteristics in shaping host inflammatory responses(10). Nevertheless, the present study did not directly investigate the molecular mechanisms responsible for these differences, and therefore these factors should be regarded as potential contributors rather than established explanations for the observed response patterns.

Magnitude of IL-6 responses differed between isolates

A major observation in this study was the difference in the magnitude of IL-6 responses between individual clinical isolates. *Citrobacter freundii* demonstrated the largest relative increase, rising from 6,190.35 pg/mL at 1×10^6 CFU/mL to 20,363.74 pg/mL at 1×10^8 CFU/mL, corresponding to a 3.29-fold increase. *Proteus vulgaris* and *Pseudomonas aeruginosa* demonstrated the next largest fold increases, at 2.24-fold and 2.20-fold, respectively.

These findings suggest isolate-specific response patterns rather than providing evidence that these bacterial species universally induce stronger inflammatory responses than other bacteria. This interpretation is particularly important because the present study included only one isolate from each bacterial species, limiting the ability to distinguish species-level effects from strain-level variation. Previous whole-blood studies have demonstrated substantial differences in cytokine-inducing capacity among bacterial species and individual clinical isolates, with marked variation in IL-6 and TNF- α production following stimulation with different bacterial isolates. These findings indicate that cytokine responses may reflect both bacterial species and strain-specific characteristics(11, 12).

The recent 2024 ex vivo whole-blood study further supports this interpretation by demonstrating that host-response profiles, including IL-6, differed sufficiently between bacterial strains to contribute to bacterial discrimination(10). Together, these observations support the view that bacterial-induced cytokine production reflects an interaction between the characteristics of the bacterial stimulus and the host immune environment rather than bacterial concentration alone.

Absolute IL-6 concentration and fold change provide complementary information

An important feature from the findings is the distinction between the highest absolute IL-6 concentration and the greatest relative increase. *Klebsiella oxytoca* produced the highest absolute IL-6 concentration in the dataset at 1×10^8 CFU/mL, reaching 24,196.03 pg/mL. In contrast, *Citrobacter freundii* demonstrated the greatest fold increase despite having the lowest IL-6 concentration at the lowest bacterial inoculum(13).

This distinction is important when interpreting cytokine-response data because absolute concentration and fold change describe different aspects of the observed response. Absolute IL-6 concentration reflects the magnitude of cytokine production measured under a specific experimental condition, whereas fold change expresses the relative difference between a reference condition and a subsequent condition. Consequently, an isolate may produce a high absolute IL-6 concentration while showing a relatively modest fold increase when its response is already elevated at the lowest inoculum. Conversely, an isolate with a relatively low initial IL-6 concentration may demonstrate a substantially greater fold increase following exposure to a higher bacterial inoculum. Recent methodological work has emphasized that fold-change measures are sensitive to the choice and characteristics of the reference and comparison values, supporting the use of fold change as a complementary descriptive measure rather than a replacement for reporting the underlying concentrations(14). In the context of bacterial

stimulation, this approach is particularly useful because clinical isolates can exhibit distinct IL-6 response profiles under comparable whole-blood experimental conditions(10).

The combined use of absolute concentrations and fold change therefore provides a more informative description of the response profiles than either measure alone. In the present study, this approach identified *C. freundii*, *P. vulgaris*, and *P. aeruginosa* as isolates with particularly large relative changes while identifying *K. oxytoca* as the isolate producing the highest absolute IL-6 concentration at the highest inoculum(15).

Heterogeneity of bacterial-induced IL-6 responses

The heterogeneity observed between isolates represents an important finding of the present study. Although twelve of the thirteen isolates had IL-6 concentrations at 1×10^8 CFU/mL that were greater than or equal to those observed at 1×10^6 CFU/mL, only seven demonstrated a progressive increase across all three concentrations. The remaining isolates exhibited non-linear or fluctuating response trajectories, indicating that the relationship between bacterial inoculum and IL-6 production was not consistent across all isolates. This variability is consistent with evidence from whole-blood stimulation studies demonstrating that different microbial stimuli can generate distinct cytokine-response profiles. In a 2023 study using standardized whole-blood stimulation, bacterial stimuli produced distinct cytokine and inflammatory-response signatures, highlighting the complexity of coordinated host responses to microbial challenge(16). Similarly, substantial heterogeneity in inflammatory cytokine production, including IL-6, has been demonstrated in whole-blood assays following stimulation with bacterial and Toll-like receptor agonists(17). Taken together, these findings support the interpretation that the magnitude and trajectory of IL-6 production may vary according to both the bacterial stimulus and the host immune environment.

Such variability is biologically plausible because whole blood provides a complex experimental environment in which multiple cellular and soluble components can participate in the response to bacterial stimulation. Monocytes and granulocytes are important contributors to cytokine production, while other blood components, including complement and platelets, can modulate inflammatory signaling and cellular interactions. Recent experimental work using human whole blood has demonstrated that selective removal of leukocyte populations can alter cytokine responses to *Escherichia coli*, highlighting the contribution of different immune-cell populations to the overall response(18). In addition, studies of bacterial stimulation in human whole blood have demonstrated that both Toll-like receptor signaling and complement activation can contribute to IL-6 production, further illustrating the interconnected nature of innate immune responses in this model(15). Thus, the IL-6 concentrations measured in the present study should be regarded as an integrated response generated within the whole-blood environment rather than as a response attributable to a single immune-cell population. This characteristic represents an important advantage of the ex vivo whole-blood model because it preserves interactions among circulating immune cells and soluble mediators that are substantially reduced or absent in assays using isolated cell populations(19).

The non-linear responses observed at the intermediate bacterial concentration should therefore not automatically be regarded as experimental error. They may reflect biological differences in the response to graded bacterial stimulation. Nevertheless, because only two donors and one isolate per bacterial species were used, the present study cannot determine whether these patterns are reproducible characteristics of individual bacterial species or reflect isolate-specific or donor-specific variation. Inter-individual variation in innate immune responsiveness is well recognized and may have contributed to differences in cytokine production observed in the present study.

Biological relevance of IL-6 in bacterial stimulation

IL-6 is a pleiotropic inflammatory cytokine that plays an important role in coordinating host responses to infection and tissue injury. Its biological effects include regulation of the acute-phase response, modulation of immune-cell activity, hematopoiesis, and coordination of interactions between innate and adaptive immune responses(20, 21). The effects of IL-6 are mediated through distinct signaling mechanisms, including classical signaling and trans-signaling, which can produce different biological outcomes depending on the cellular and inflammatory context(21). Excessive or dysregulated IL-6 signaling may contribute to sustained inflammation and tissue pathology, whereas appropriately regulated IL-6 activity is important for effective host defense and immune regulation(21, 22). Therefore, IL-6 concentrations should be interpreted in relation to the experimental and biological context rather than considered as a direct measure of disease severity or inflammatory damage.

The present findings should nevertheless be interpreted within the context of an ex vivo experimental system. The IL-6 concentrations measured in the culture supernatants represent cytokine production under controlled laboratory conditions and should not be directly equated with circulating IL-6 concentrations in patients. In vivo cytokine concentrations are influenced by the magnitude and localization of the infectious stimulus, recruitment and activation of immune cells, tissue-specific responses, cytokine signaling and clearance, and systemic organ function, among other regulatory processes that cannot be fully reproduced in an ex vivo assay(10, 19). Importantly, experimental studies comparing ex vivo cytokine responses with in vivo inflammatory responses have demonstrated that cytokine production measured after ex vivo stimulation may not reliably predict the magnitude of the corresponding in vivo response, emphasizing the limitations of extrapolating ex vivo findings directly to clinical conditions(23). Therefore, the IL-6 concentrations observed in the present study should be interpreted as measures of the capacity of the tested bacterial isolates to stimulate an inflammatory response within the ex vivo whole-blood environment rather than as direct estimates of circulating IL-6 concentrations or clinical inflammatory severity.

This distinction is particularly important when discussing clinical implications. The present results demonstrate differences in cytokine responses under controlled laboratory conditions but do not establish that isolates producing greater IL-6 responses in vitro necessarily cause more severe disease in infected patients.

Relevance of the whole-blood model

The ex vivo whole-blood model provides a practical approach for investigating host responses to clinically derived bacterial isolates while retaining interactions between multiple blood-cell populations. Unlike assays based on a single purified immune-cell population, whole blood provides a more integrated experimental environment in which bacterial stimuli encounter monocytes, neutrophils, lymphocytes, platelets, complement, and soluble inflammatory mediators.

Recent research has demonstrated the utility of ex vivo whole-blood models for characterizing host responses to clinically relevant bacterial stimuli. In a 2024 study, Chick and colleagues used an ex vivo whole-blood model to investigate multiple host-response parameters following stimulation with eight bacterial strains representing three species and two genera. The investigators identified distinct temporal response profiles across the measured parameters, including IL-6, and found that IL-6 contributed to discrimination between bacterial genera and species. These findings provide further evidence that cytokine responses generated in whole blood can vary according to the bacterial stimulus and may contain information useful for characterizing differences between bacterial strains and species(10).

The present study extends this general approach by applying three standardized bacterial inoculum concentrations to clinical isolates recovered from routine diagnostic specimens. The design therefore provides a simple framework for describing how cytokine responses change as the bacterial stimulus is increased.

Interpretation of the observed responses in relation to bacterial characteristics

The inclusion of both Gram-negative and Gram-positive clinical isolates provided an opportunity to observe variation in IL-6 responses among bacteria with distinct structural characteristics. Gram-negative bacteria possess an outer membrane containing LPS, whereas Gram-positive bacteria lack an outer membrane and contain cell-wall components such as peptidoglycan and lipoteichoic acids. These microbial structures can be recognized by different, although partly overlapping, innate immune-sensing pathways and may consequently influence the magnitude and profile of cytokine production(10, 15).

Although the present dataset was too small to support a formal comparison between Gram-positive and Gram-negative organisms, the observed heterogeneity is consistent with previous whole-blood studies demonstrating species- and strain-dependent differences in cytokine-inducing capacity. In an experimental study involving 49 clinical bacterial isolates, Gram-negative organisms generally induced higher IL-6 concentrations than Gram-positive organisms; however, substantial differences were also observed between individual bacterial species and strains(13). More recent ex vivo whole-blood research has likewise demonstrated distinct IL-6 response profiles following stimulation with different bacterial strains, further supporting the importance of bacterial-specific characteristics in shaping host inflammatory responses(10).

Nevertheless, it would be inappropriate to attribute the IL-6 responses observed in the present study solely to Gram classification. Gram status represents a broad structural classification and does not capture the considerable variation in surface components, virulence-associated characteristics, and host-recognition pathways that may occur between individual species and strains. The present findings are therefore better interpreted as demonstrating heterogeneity among individual clinical isolates, with bacterial structural characteristics representing one of several possible contributors to the observed IL-6 response patterns. Although exploratory, these findings demonstrate the utility of standardized ex vivo whole-blood assays for characterizing inflammatory responses to clinically relevant bacterial isolates and may provide a useful framework for future immunological and translational investigations.

Implications for future research

The findings provide several directions for future investigation. First, the isolates demonstrating the largest fold changes, particularly *C. freundii*, *P. vulgaris*, and *P. aeruginosa*, could be prioritized for more detailed mechanistic studies. Such studies could investigate whether their enhanced responses are associated with particular bacterial surface structures, virulence-associated factors, or host-recognition pathways.

Second, future studies should include a larger number of isolates per bacterial species. This would help determine whether the response patterns observed in the present study are reproducible species-level characteristics or properties of individual clinical strains.

Third, incorporating additional cytokines such as TNF- α , IL-1 β , IL-10, and chemokines would provide a broader characterization of the inflammatory response. The recent whole-blood literature demonstrates the value of measuring multiple host-response parameters rather than relying on a single cytokine endpoint.

Finally, increasing the number of healthy donors would allow future studies to examine donor-to-donor variation more systematically. This would be particularly important if the ultimate objective is to determine whether specific bacterial isolates consistently induce distinct immune-response signatures across different host backgrounds.

Strengths and limitations of the study

The study's major strength is the use of a standardized ex vivo whole-blood model with clinically derived bacterial isolates and graded inoculum concentrations, allowing direct characterization of isolate-specific and dose-dependent IL-6 responses under controlled experimental conditions. Inclusion of both Gram-positive and Gram-negative isolates further captured the heterogeneity of inflammatory responses observed among clinically relevant bacteria. The study was exploratory in nature and included a limited number of isolates and healthy donors, which provides an important foundation for generating descriptive response patterns. The assessment of IL-6 as a key inflammatory mediator provided focused insight into early host responses, while future studies can build on these findings by incorporating larger isolate collections, additional donors, and broader cytokine panels. In addition, the study did not include a

standardized positive immune stimulant such as LPS, which could have provided a useful reference for comparing cytokine responses across bacterial stimuli.

Conclusions

Taken together, the findings demonstrate that the clinical bacterial isolates examined in this study produced distinct IL-6 response profiles in the ex vivo whole-blood model. Increasing bacterial concentration was generally associated with higher IL-6 production, although response patterns varied among isolates.

This study demonstrates that clinically derived bacterial isolates generate distinct IL-6 response profiles in an ex vivo whole-blood model. Increasing bacterial inoculum was generally associated with increased IL-6 production; however, the magnitude and trajectory of responses varied among isolates. These findings highlight the influence of both microbial stimulus intensity and isolate-specific characteristics on early innate immune responses. Given the exploratory design, larger studies incorporating multiple isolates per species, additional donors, and broader cytokine profiling are required to validate these observations and investigate underlying mechanisms.

Abbreviations

CFU — Colony-forming units

CO₂ — Carbon dioxide

ELISA — Enzyme-linked immunosorbent assay

IL-6 — Interleukin-6

KCMC — Kilimanjaro Christian Medical Centre

KCRI — Kilimanjaro Clinical Research Institute

LPS — Lipopolysaccharide

OD — Optical density

PAMPs — Pathogen-associated molecular patterns

PBS — Phosphate-buffered saline

PRRs — Pattern-recognition receptors

SPSS — Statistical Package for the Social Sciences

TLRs — Toll-like receptors

Declarations

Ethical Considerations

Ethical approval for the study was obtained from the Kilimanjaro Christian Medical University College Research Ethics and Review Committee before commencement of the study, under ethical clearance certificate number UG 104/2024. Written informed consent was obtained from all healthy blood donors before sample collection. Participant confidentiality was maintained throughout the study by assigning unique identification codes, and all laboratory procedures complied with institutional biosafety and ethical guidelines.

Clinical Trial Number

Not applicable

Consent of Publication

Not applicable. This manuscript does not contain data from any individual person, including individual identifying details, images, or videos.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MHI conceived and designed the study, conducted the laboratory experiments, collected and analyzed the data, interpreted the findings, and drafted the manuscript. DM contributed to the study design, provided scientific supervision, supported interpretation of the findings, and critically reviewed and revised the manuscript. Both authors read and approved the final manuscript.

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Figures

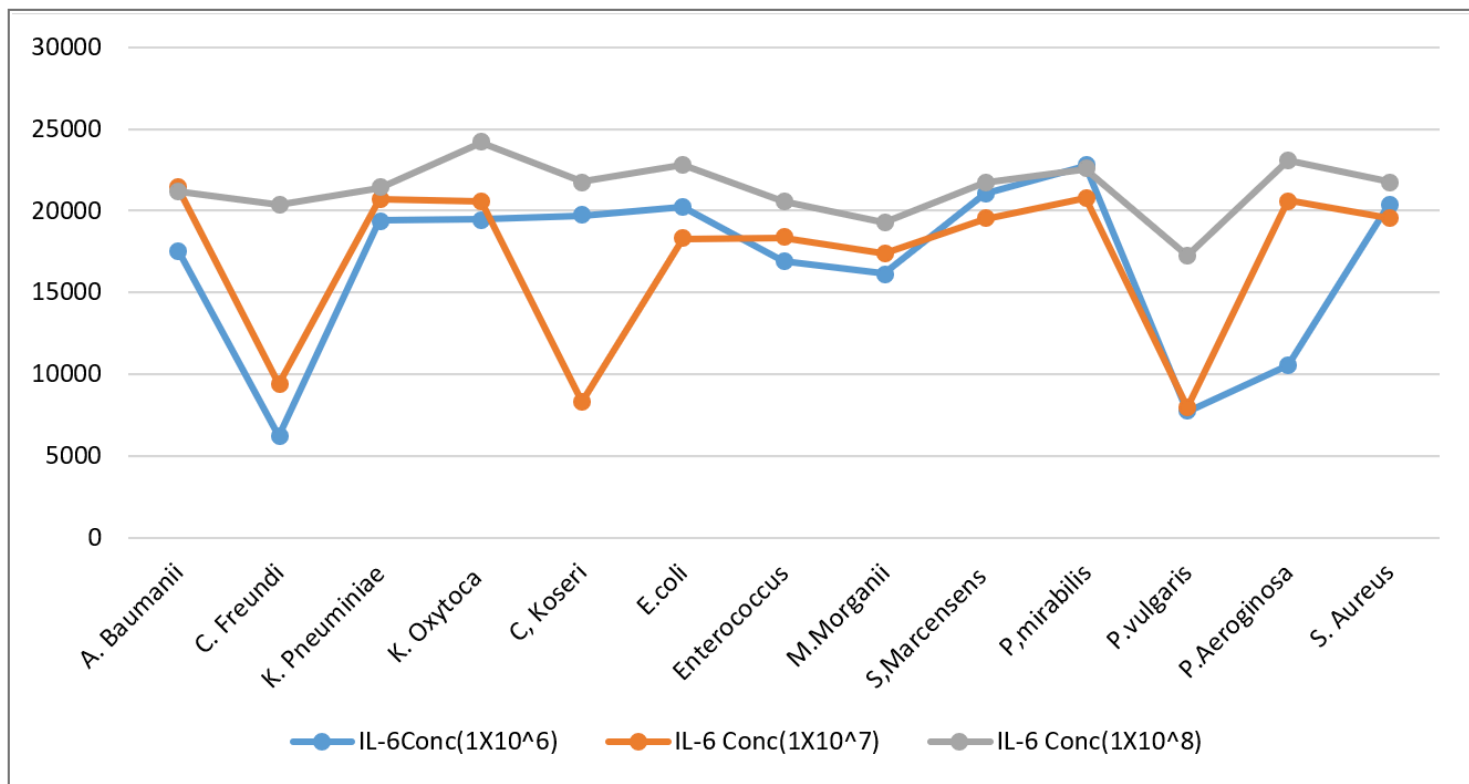


Figure 1

Dose-dependent IL-6 responses induced by clinical bacterial isolates at increasing inoculum concentrations

Note: Data represent IL-6 concentrations (pg/mL) measured following ex vivo whole-blood stimulation with heat-inactivated bacterial isolates at three inoculum concentrations (1×10^6 , 1×10^7 , and 1×10^8 CFU/mL). Each isolate was tested using whole blood from two healthy adult donors. CFU, IL-6.