Prevalence, Distribution, and Diagnostic Testing for *Legionella*, and Growth Curves and Immune Responses of Clinical Isolates of *Legionella*: A Scoping Review and an *in-vitro* study

By

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A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfillment of the requirements of the degree of

Master of Science

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Abstract

Background: *Legionella* spp. is an underrecognized etiology of pneumonia, capable of causing severe disease and occurring more frequently in people living with HIV and over the age of 45. Studies suggest that non-*pneumophila* serogroup 1 *Legionella* is underdiagnosed. Research and diagnostics for species and serogroups other than *Legionella* pneumophila serogroup 1 are limited due to reliance on urinary antigen for diagnostics.

Methods: A scoping review was conducted to evaluate the prevalence and incidence of *Legionella* stratified by species and serogroups, and the methods used to detect *Legionella*. Articles were extracted from several databases and independently screened by 2 researchers. U937 cells were infected with *L. pneumophila* Philadelphia-1 and clinical strains of *L. bozemanae*, *L. dumoffii*, *L. micdadei*, and *L. pneumophila* from a tertiary care hospital in Winnipeg, Manitoba. Intracellular growth of *Legionella* was evaluated by a colony-forming unit assay (CFU). Cell culture supernatants were evaluated for Eotaxin, FGF-2, fractalkine, GM-CSF, IFN-α2, IFN-γ, IL-1β, IL-6, IL-8, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IP-10, MCP-1, MCP-2, RANTES, TGF-β, and TNF-α.

Results: 31 of 3449 articles met the inclusion criteria for the review. The most common species found were *L. pneumophila*, *L. longbeachae*, and unidentified *Legionella* species in 1.4%, 0.9%, and 0.6% of total pneumonia cases. Nearly 50% of Legionnaires' disease cases are caused by species not detected by first-line diagnostics. NAT-based techniques were more likely to detect *Legionella* than non-NAT-based techniques. U937 cells increased expression of TGF-β when infected with *Legionella bozemanae*; decreased GM-CSF when infected with *Legionella dumoffii*; increased expression of MCP-2 when infected with *Legionella micdadei*; decreased expression of FGF-2, GM-CSF, and IL-8 when infected with *Legionella pneumophila*; and increased FGF-2 when infected with *Legionella pneumophila* Phildadelphia-1.

Conclusions: *Legionella* detection is hampered by a lack of application of broader or pan-*Legionella* diagnostics. Our findings provide new insights into differential cytokine responses elicited by *Legionella* species, highlighting the need for further research into specific mechanisms involved in the responses.

Acknowledgements

I am grateful to all of the people involved in helping me in my time in MMID. This degree is not the result of a single person's work, but of the cumulative efforts of many. First and foremost, I would like to thank my advisors Drs. Zulma Rueda and Yoav Keynan for their support, patience, and guidance throughout the course of my program. They opened my eyes to many different aspects and perspectives in research, providing opportunities for me to learn skills that I otherwise would not have learned.

I would also like to thank Dr. Mariana Herrera, Dr. Sandra Gonzalez, Dr. Barbara Porto, Dr. Xiaojin Yao, Toby Le, and Natasha Hollett for providing me guidance whenever I had questions and their expertise on topics ranging from project design to troubleshooting to statistical analyses.

I'd like to thank the administrative staff from MMID, Angela Nelson for answering any and every administrative question I have, Leah Buermeyer for helping set up rooms for meetings and practice presentations, Anh Pham for helping me with ordering lab supplies and passing the time.

To my committee members Drs. Jason Kindrachuk and Ann Karen Brassinga I appreciate the feedback and support provided to me. And I'd like to extend further gratitude to Dr. Brassinga and her lab, especially Christopher Graham, Teassa MacMartin, and Tiffany Penner, for accepting me and teaching me cell culturing techniques and how to work with *Legionella*.

To my friends Erwin Taguiam, Edwin Nguyen, and David Lam, I appreciate the time we've spent together and the support you have all provided through the years.

Author contributions

Ryan Ha performed the research for this master's thesis and wrote the first draft of the thesis and articles. The disaggregated contributions of the published and submitted articles can be found below:

Chapters 1, 4, 5, 6, 7:

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Chapter 2:

Conceptualization: Ryan Ha, Yoav Keynan, and Zulma Vanessa Rueda; methodology: Ryan Ha and Zulma Vanessa Rueda; investigation: Ryan Ha, Ashley Heilmann, and Zulma Vanessa Rueda; validation: Yoav Keynan and Zulma Vanessa Rueda; formal analysis: Ryan Ha completed the first draft of the analysis, then all authors contributed to additional analysis and interpretation; data curation: Ryan Ha and Ashley Heilmann; writing—original draft: Ryan Ha with some contributions from Ashley Heilmann; writing—review and editing: Sylvain A. Lother, Christine Turenne, David Alexander, Yoav Keynan and Zulma Vanessa Rueda; resources: Zulma Vanessa Rueda; visualization: Ryan Ha, Ashley Heilmann, Yoav Keynan, and Zulma Vanessa Rueda; supervision: Yoav Keynan and Zulma Vanessa Rueda; project administration: Zulma Vanessa Rueda; funding acquisition: Zulma Vanessa Rueda and Yoav Keynan. All authors have read and agreed to the published version of the manuscript.

Chapter 3:

Conceptualization, Ryan Ha, Yoav Keynan, and Zulma Vanessa Rueda; methodology, Ryan Ha, Mariana Herrera, and Sandra Gonzalez; formal analysis, Ryan Ha with guidance and mentoring from Mariana Herrera, and Zulma Vanessa Rueda; investigation, Ryan Ha; resources, Yoav Keynan and Zulma Vanessa Rueda; data curation, Ryan Ha; writing—original draft preparation, Ryan Ha; writing—review and editing, Ryan Ha, Mariana Herrera, Sandra Gonzalez, Yoav Keynan, and Zulma Vanessa Rueda; visualization, Ryan Ha; supervision, Yoav Keynan and Zulma Vanessa Rueda; project administration, Zulma Vanessa Rueda; funding acquisition, Zulma Vanessa Rueda. All authors have read and agreed to the submitted version of the manuscript.

Preface

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Abbreviations

Ank	Ankyrin
ASH	ASPM, SPD-2, Hydin
BALF	Bronchioalveolar lavage fluid
BCYE	Buffered yeast charcoal extract
BMDM	Bone marrow-derived macrophage
CAP	Community-acquired pneumonia
CDC	Center for Disease Control
CsrA	Carbon storage regulator A
DFA	Direct fluorescent antibody
ELISA	Enzyme-linked immunosorbent assay
FGF-2	Fibroblast growth factor-2
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GTP	Guanosine triphosphate
HIV	Human immunodeficiency virus
IFN	Interferon
IL	Interleukin
IP	Interferon-γ-induced protein
JAK	Janus kinases
LD	Legionnaires' disease
LPS	Lipopolysaccharide
LCV	*Legionella*-containing vacuole
LLOD	Lower limit of detection
LPSG1	*Legionella pneumophila* serogroup 1
LRT	Lower respiratory tract
MCP	Monocyte chemoattractant protein
MavN	More regions allowing vacuolar colocalization N
MM6	Mono-Mac-6
MOMP	Major outer membrane protein
NAAT/NAT	Nucleic acid amplification tests
NAIP	Neuronal apoptosis inhibitory protein
NCCID	National Collaborating Center for Infectious Diseases

OCRL	Oculocerebrorenal syndrome of Lowe
PBMC	Peripheral blood monocytic cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
PICOT	Population, intervention/exposure, comparator, outcome, timeframe
PLHIV	People living with HIV
PMA	Phorbol 12-myristate-13-acetate
Rab	Ras-associated protein
Raf	Rapidly accelerated fibrosarcoma
RANTES	Regulated upon activation, normal T-cell expressed and secreted
Ras	Rat sarcoma virus
Rho	Ras homolog
RPMI	Roswell Park Memorial Institute
RT	Real-time
SBT	Sequence-based typing
SdhA	Succinate dehydrogenase A
SG	Serogroup
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activators of transcription
T1SS	Type 1 secretion system
T2SS	Type 2 secretion system
T4SS	Type 4 secretion system
TGF	Transforming growth factor
Th	T helper
TNF	Tumour necrosis factor
UAT	Urinary/urine antigen test
VipA	VPS inhibitor protein A
Vps29	Vacuolar protein sorting 29
WGS	Whole genome sequencing

Preamble

This work is in the format of a sandwich thesis, comprised of a mix of published articles as part of my master's research project and an additional article submitted for publication also derived from my master's project. Chapters 1 is a generalized introduction providing context behind chapters 2 and 3, each consisting of an independent publication. As of the writing of this thesis, chapter 3 is under preparation for publication and has not yet been. Sections 4 to 6 comprise the general discussion, conclusion, and future steps.

Chapter 1: Introduction

1.1 Diversity and Ecology of *Legionella* spp.

Legionella is a family of gram-negative bacteria that live primarily in aquatic environments including both natural and man-made water systems, as well as soil. Inhalation of *Legionella*-contaminated aerosols is the primary means of infection. Currently, there are 65 known species of *Legionella* and 25 have been reported to cause disease (1). Clinically, the most common species are *L. pneumophila*, *L. longbeachae*, *L. dumoffii*, *L. micdadei*, *L. bozemanii*, and *L. anisa* (2).

In nature, *Legionella* can be found in biofilms or occupying eukaryotic hosts as a means of survival and a source of nutrients. Within anthropogenic water systems, biofilms serve as a niche for the survival of *Legionella*. To replicate in these environments a host, typically an amoeba, is required (3,4). This is a result of lower nutrient content in anthropogenic water systems (3). *Legionella's* host range consists of various species of amoeba and mammals such as cattle and humans (5–7). The host range of *Legionella* is united by a shared physiological niche found across distantly related hosts as exemplified by its affinity for establishing infections within mammalian phagocytic host cells, thus allowing *Legionella* to spread between taxonomically distinct hosts, incorporating new genes to drive its evolution (6,8). It has even been suggested that the composition of a biofilm's microbiome is a key factor in *Legionell*a's ability to thrive (9).

1.2 Epidemiology

The impact that each species has on public health is locale dependent. *L. pneumophila* is the predominant etiological agent in North America, where most infections are reported to be caused by *L. pneumophila* serogroup 1. In 2022, Public Health Ontario reported 360 cases of Legionellosis (Legionellosis is any *Legionella* infection, Legionnaires' disease (LD) is pneumonia caused by *Legionella*) at an incidence rate of 2.4 per 100,000 with 268 hospitalizations and 21 deaths (10). In 2021, the Centers for Disease Control and Prevention (CDC) reported an incidence rate of approximately 2.5 per 100,000 with higher rates in black persons, males, older adults, Northeast and Midwest regions, and in the summer and fall. Increases in incidence have been reported globally, however, many cases go undetected (11–15). *L. longbeachae* is of significant concern in Australia and New Zealand where *L. pneumophila* is present at a lower frequency compared to what is reported in North America (16–19).

Overall, the overreliance of clinical diagnostics on urinary antigen tests (UAT) as the primary diagnostic tool narrows the ability to identify non-serogroup 1 *Legionella* limiting the understanding of the role non-

pneumophila Legionella species play in clinical disease. The higher detection rates of *L. longbeachae* infections in Oceania are attributed to the use of polymerase chain reaction (PCR) as part of the standard diagnostic procedure for legionellosis (17).

Some species are observed to have been identified in the context of coinfections with copathogens such as *L. pneumophila* and *L. anisa* with *Mycobacterium tuberculosis*, and *L. bozemanae* with *Pneumocystis jirovecii* (20). *Legionella* infections in immunocompromised individuals appear to be associated with less common species (20,21). In people living with HIV (PLHIV), individuals who were infected with *Legionella* suffered higher rates of mortality than those who did not (53% and 20%, respectively) (20). Reduced immune function, whether by endogenous causes (e.g., genetic defects) or exogenous causes (e.g., immunosuppressive treatment regimens or positive human immunodeficiency virus (HIV) status) can further increase the risk of *Legionella* infection, among other respiratory pathogens. The reduced immune function also worsens outcomes and severity in a population already at a heightened risk. The adaptive immune system, namely CD4+ T-cells are depleted during untreated HIV infection, interfering with the overall immune response (22). As a consequence of reduced CD4+ T-cell function, overall immune activation is disrupted, abating the activity of B-cells, CD8+ T-cells, basophils, eosinophils, neutrophils, and macrophages (23).

Individuals are at an increased risk of contracting a *Legionella* infection if they are 50 years of age or older, immunocompromised/immunosuppressed, smoke, male sex or have other underlying chronic health conditions (14,24,25). As life expectancy is increasing in many parts of the world, it can be expected that the incidence of *Legionella* infections will increase. The average age of legionellosis patients has decreased since the late 1990s while the overall case rate increased (11). In the past, most cases occurred in individuals aged 65-74, while most cases now occur in individuals aged 55-64 (11). This may indicate a change in epidemiology, however, this change may also stem from evolving diagnostic practices driving improved case identification or factors such as aged water systems (26–29).

An increase in *Legionella* infections has been noted over the past 2 decades. This change is thought to be, in part caused by the rise in average global temperatures (11). It has been noted that *Legionella* cases typically occur between late spring and early autumn when temperatures are higher (11,15). *Legionella*'s seasonality coincides with that of amoebic blooms, such as during the wet pre-monsoon period in Pakistan, suggesting that the environmental *Legionella* population is tied to the amoebic population (30,31). Furthermore, *Legionella* is the most common non-gastrointestinal pathogen following tropical storms, which occur more frequently in the seasons during which *Legionella* infections are more common

(11,15,32,33). This occurs as a result of untreated water being mixed with water reservoirs, contaminating them with amoeba and *Legionella*. The burden this imposes on the healthcare system, especially in a post-disaster effort, encourages studies in preventative measures to protect systems from contamination.

1.3 Clinical Presentations

While *L. pneumophila* is regarded as the species of utmost clinical concern, several other *Legionella* species have been found to pose significant public health threats, causing similar manifestations and mortality rate (20,34).

Collectively known as legionellosis, *Legionella* spp. infections present as two main febrile syndromes: Pontiac fever, a mild self-resolving illness featuring fatigue, headache, malaise and muscle aches without pneumonia; and Legionnaires' disease, a potentially severe disease with cough, shortness of breath with a range of severity and up to ¼ of all cases requiring ICU admission and with associated significant mortality rates estimated to range from 4-18% (35,36). The incidence of Pontiac fever is unclear, as the disease is mild enough that cases go unreported (37). *Legionella* infections have also been associated with an absence of sputum production, low serum sodium concentrations (132-133mmol/L), high levels of lactate dehydrogenase (242-465U/L), high levels of C-reactive protein (241-328mg/L), high levels of creatinine (95-101µmol/L), and low platelet counts ($167x10^9$-$219x10^9$/L) (38–43). The non-specific symptoms and relatively mild illness during Pontiac fever and non-specific symptoms during Legionnaires' disease contribute to difficulties in initial diagnoses.

Radiographical manifestations include patchy unilobar infiltrate, ground glass opacities, multilobar or segmental patchy infiltrates, favouring the lower lobes with occasional pleural effusion and mediastinal lymphadenopathy without pneumothorax or cavitation (25,44–46). Radiographic findings are not seen to have a strong association with outcomes (44). Computerized tomography shows multilobar or multisegmented well-circumscribed air-space disease with ground-glass opacities that can progress to confluent infiltrates (25).

As with many other pathogens, extrapulmonary infections are rare, albeit possible (47). Some reports have even stated that *Legionella* can cause sputum to appear yellow or orange (48–51). This is owing to tyrosine from the pulmonary epithelia being processed by *Legionella* (48,50,51). Extrapulmonary symptoms include fever, loss of appetite, headache, malaise, lethargy, muscle pain, diarrhea, and confusion (24). In extreme cases, presentations may include lymphadenopathy, panniculitis, hepatitis, atrio-ventricular block, arthritis, prosthetic valve endocarditis, and myocarditis, which can develop into

septic shock and organ failure if left untreated (52–60). Mortality occurs in 10% of patients, further increased to 25% in individuals with several risk factors and 45% in smokers (60–62). Cases may also require intensive care if severe enough (63,64).

1.4 Diagnosis

Diagnosis of *Legionella* is complicated for a multitude of reasons. The clinical presentation of pneumonia is similar between bacterial and viral etiology, which limits the clinical diagnosis of *Legionella* as the etiological agent in many cases. Diagnostic tests used when *Legionella* is suspected include urinary antigen tests (UAT), culture, immunofluorescent stains, serology, and nucleic acid amplification tests (NAAT). However, clinical tests are often limited to UAT and culture, forgoing other methods because of the limited sensitivity of culture, the requirement for enriched media, and the costs or availability of other tests. UAT has a narrow spectrum of targets, limiting detection to *L. pneumophila* serogroup 1 (LPSG1). Culturing as a diagnostic is exceptionally lengthy and also delivers a high volume of false negatives. Samples used in culturing, bronchioalveolar lavage fluid (BALF) and sputum, are hard to obtain, and isolation is impossible in Pontiac fever patients (37–39). A 2022 Legionellosis outbreak in Argentina further proved that UAT and culture are insufficient for detecting *Legionella* on their own and require the coalescence of traditional methods with modern molecular techniques and better prevention strategies (65).

Serological testing is recommended to include a mixture of IgA, IgG, and IgM testing and is often detectable 3-4 weeks post-infection but may take over 10 weeks (66,67). A diagnostic reading requires that a reciprocal antibody titer increase of 4-fold to be ≥128 (66). However, cross-reactivities for non-*L. pneumophila* serogroup 1 and even other bacteria have been reported to show seroconversion for *L. pneumophila* serogroup 1 antibodies (66,68,69).

Countries such as Australia, and New Zealand have found success using broader diagnostic testing, including NAAT in addition to UAT and culture for *Legionella* diagnosis. These countries also report higher rates of non-LPSG1 infections, exemplifying the merit in the increased use of PCR (17,18,70). In North America, NAAT is used regionally, but not ubiquitously (71). Japan has also seen success using a loop-mediated isothermal amplification technique, which has been approved for insurance since 2011 (72–74). When used more routinely, molecular diagnostic tests have been reported to have higher rates of detection than standard diagnostic techniques compared to culture (75,76).

1.5 Treatment

Legionella is resistant to most antibiotics, owing to its biphasic lifecycle in which it adopts a spore-like cyst lifestyle to drastically slow down biological processes without entering a state of full dormancy as would be seen in spores and biofilm inhabitation in environmental and manmade water systems (77,78). Outside of the cyst form, much of its lifespan during infection is spent within host cells, necessitating the use of antimicrobials that attain therapeutic concentrations within eukaryotic cells. Thus, effective treatments are limited to drugs from the classes of macrolides, quinolones, fluoroquinolones, and tetracyclines, sometimes complemented with rifampin or sulfonamides (79–82). Antimicrobial susceptibility testing is not routinely performed because of difficulty growing the organism and a lack of available standardized methods, hence, therapy is empiric. Delays in *Legionella*-appropriate treatment have been associated with worsened prognoses; thus, diagnosis must be carried out quickly (63,64,83,84).

1.6 Immunology and Pathogenesis

1.6.1 Anti-*Legionella* Immunology

In the environment, *Legionella* finds hosts in numerous amoeba species, facilitating the need for distinct adaptations to establish in different eukaryotic hosts (3,4). The physiological similarities between amoeba and macrophages are the drivers behind the evolutionary changes enabling occupancy within multicellular eukaryotes such as humans (85). While differences are present between each *Legionella* species, their genomes encode common features that enable establishment within eukaryotic hosts such as a type 1 secretion system (T1SS), type 2 secretion system (T2SS), type 4 secretion system (T4SS), and various eukaryote-like proteins (86–90). The expression of the T2SS and T4SS are limited to the stationary phase, during which *Legionella* is most infection permissive.

During infection, *Legionella* spp. primarily reside in phagocytic cells such as macrophages. Macrophages are the primary phagocytic cells and play both inflammatory and anti-inflammatory roles, and are one of the key mediators of the innate immune system. M1 macrophages are proinflammatory, responding to and producing proinflammatory cytokines such as TNF-α and IFN-γ to phagocytose foreign material during the initial stages of infection. Phagocytosed material is destined for degradation by exposure to reactive oxygen species and lytic enzymes before being presented to adaptive immune cells (91). M2 macrophages can clear debris from apoptotic cells and reduce inflammation. This anti-inflammatory activity has been seen to occur in macrophages exposed to TGF-β. M2 macrophages shift the IL-10:IL-12 balance to favour the IL-10-mediated inhibition of inflammation (92). In *L. longbeachae* infections it has been shown that

resident immune cells are ineffective at clearing infection, instead facilitating bacterial growth, while infiltrating cells cleared the infection via the IL-18R axis (93).

Intracellular *Legionella* in the growth phase is non-motile and minimally virulent, undergoing various physiological changes distinct from those seen when grown without a host (77,94,95). This alternative form has been referred to as the mature intracellular form, which is enriched to display a phenotype exhibiting significantly increased resistance to various stressors and increased virulence compared to the *in vitro* equivalent (77,96). Temporal and spatial trigger of post-exponential virulence-associated regulatory cascades by *Legionella pneumophila* after bacterial escape into the host cell cytosol (97).

Further macrophage recruitment is essential to the pathogenesis of *Legionella*. This process depends on several chemotactic and proinflammatory factors, such as TNF-α, IFN-γ, IP-10, MCP-1, and MCP-2, all playing a role in the recruitment and activation of monocytes and macrophages (98–102). Upon invasion of a phagocyte, *Legionella* hijacks the host machinery to prevent autophagy to aid in its survival while siphoning nutrients from its host (103). Katagiri et al. observed the induction of pyroptosis by neuronal apoptosis inhibitory protein (NAIP) in *Legionella pneumophila*-infected mouse macrophages, where NAIP would normally inhibit apoptosis (104). The role of NAIP in *Legionella* and other bacterial infections has been previously described, with some authors even noting that the NAIP copy number impacts the outcome (105–107).

In contrast to immune responses elicited by *L. pneumophila*, *L. longbeachae* has been reported to cause immunologically silent infections in mice, with some corroborating evidence seen in human neutrophils (108). This may be owing to the formation of a phagocytosis-resistant capsule causing immune evasion in *L. longbeachae* that has not been observed in *L. pneumophila*(108,109).

1.6.2 *Legionella* pathogenesis

This intracellular growth involves vacuoles called *Legionella*-containing vacuoles (LCV), used as a form of immune evasion. Within the LCV, *Legionella* secretes effector proteins through the T4SS to perform tasks such as preventing phagolysosome fusion, cell trafficking disruption, and siphoning nutrients from the host cell (109). Most *Legionella* research so far has been limited to *Legionella pneumophila* serogroup 1 (LPSG1); however, other less virulent species and serogroups that have been studied appear to have similar mechanisms (110–112). Some major virulence factors are notably not ubiquitous within the genus, such as the flagella and T2SS or the capsule formed by *L. longbeachae* (113). In this section, the outlined mechanisms are those known to be employed by LPSG1, but some are common throughout the genus.

Under starvation, *Legionella* shifts to a virulent, flagellated (depending on species) spore-like form. The regulation of this process is dictated by several regulatory proteins, metabolites, and non-coding RNA (114–116). Upon entering a host cell, stress responses linked to LetAS and CsrA (Carbon storage regulator A) allow for T4SS-associated effector proteins to be produced and released into the host. *L. pneumophila* is notable for encoding over 300 effector proteins that are distributed via its T4SS, increasing up to over 18,000 across the genus (8,117). However, most of these effectors are either uniquely found within *L. pneumophila* or scattered across a few other species and not universally found in *Legionella*, however, functional homologs have been found across species (8,111,117,118). These effectors may be necessary for virulence, but individual knockouts are not detrimental to infectivity and almost all effectors are not important enough to be essential (85,117).

Only 9 effectors are considered to be core proteins across the genus (111,117). These effectors have functionally redundant orthologs in each species and several can be exchanged while conserving function (111,119). Among these core effectors, not all are secreted, indicating chaperonin or post-translation processing activity (111). These interactions with host biomolecules for immune evasion and host hijacking are presumed to be the purpose of a wide arsenal of eukaryote-like proteins released through the T4SS.

1.6.3 Evolution of *Legionella* pathogenesis

The intracellular behaviour of *Legionella* drives its evolution, as made evident by its eukaryote-like proteins and eukaryote-like motifs, much like viral evolution (110,120–122). While this array of eukaryote-interacting proteins has resulted in the ability to survive within eukaryote hosts, variation is seen in the factors required for each host, even within the same phylum (118). Interestingly, *L. longbeachae*, unique among pathogenic *Legionella* for being soil-borne, exhibits proteins and motifs that are uniquely found in plants or are plant-interacting (110). Presumably, these eukaryote motifs result from its intracellular lifestyle and the rich biodiversity of *Legionella*'s aquatic environments. *Legionella* encodes several putative genes for the biosynthesis of amino acids that it is auxotrophic for, indicating that these genes are either expressed under unknown conditions or are remnants of old pathways from before *Legionella* became a pathogen (123). The physiological redundancies at the cellular level in eukaryotes have allowed *Legionella* to adapt to several hosts. Thus, the opportunity allotted to *Legionella* by its survival in protozoa has been a crucial step in its evolution as a pathogen.

Legionellae have acquired genes from a myriad of hosts, marked by domain-specific regions from plants, animals, fungi, and archaea (8). *Legionellae* are not currently known to have any plant or fungal hosts;

thus, it is unclear how plant- and fungi-specific domains have been acquired. While the acquisition of these genes suggests that hosts may share a taxonomic kingdom, they may have been obtained from exogenous DNA in organism-rich environments. Presumably, these proteins act as virulence factors, inducing favourable conditions to enable their intracellular growth as they are often distributed by the T4SS, which is only expressed during infection. Some protein domains acquired by *Legionella*, such as C-terminal alliinase and caleosin, may also provide a competitive edge over other pathogens within amoebic hosts or in the environment (8). Eukaryote domains are abundant in *Legionella*, many even within eukaryote-like proteins (8). This suggests that these domains have either been co-opted by *Legionella*, for novel virulence factors or constitute non-functional fusion proteins resulting from unsuccessful recombination and that many have been recently incorporated into the *Legionella* genome.

While all species of *Legionella* have a T4SS, non-*pneumophila Legionella* species possess a significantly smaller pool of effectors (124). Genetic exchange of these effectors between species occurs infrequently, making each species' T4SS repertoire almost entirely distinct from the others (117,125). The basis for *Legionella*'s survival and host modulation lies within its T4SS and its effectors. *Legionella* has been recorded to possess T1SS and T2SS, but while they are recognized as being involved in virulence, much less is known about these systems and their effector proteins (89,90,126–129). Within phagocytes, the core functions of the T4SS secretome include inhibition of phagosome maturation, hijacking of endocytic and secretory pathways, upregulated ubiquitination, host signaling disruption, post-translational modification disruption, and apoptosis inhibition, ensuring the survival of internalized *Legionella* (121,122). Thus, the T4SS is the main driver of *Legionella*'s virulence, secreting effector proteins based on life stage 27). In contrast to diversity in the effectors, *Legionella*'s T4SS is highly conserved across the genus (8). This secretome includes proteins that prevent phagolysosomal fusion, macromolecule trafficking, and regulation of protein synthesis, many of which contain regions normally found in eukaryotes (8,86,130–132). Many proteins secreted through *Legionella*'s T4SS contain domains otherwise exclusively found in eukaryotes, highlighting their importance for growth and survival within eukaryote hosts (8,131–134).

As with many other pathogens, *Legionella* can modulate the host immune system to favour its own survival. While disrupting recognition by the host, it also requires a method of obtaining resources for growth and replication. An important part of the evasion mechanism is by forming vacuoles using eukaryote host lipids capable of safely housing *Legionella* within host cells, a process involving vacuolar fusion and lipid synthesis (135–138). Interestingly, *Legionella* has not been recorded to be capable of *de*

novo synthesis of eukaryote lipids, and several species encode proteins that can produce phosphatidylcholine from exogenous choline or by methylating phosphatidylethanolamine (135,139,140). Typical bacterial membranes consist of phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin (136). However, *Legionella* membranes also consist of the eukaryote lipids, phosphatidylcholine and diphosphatidylglycerol (135,136,139–144). Furthermore, the protein SdhA (succinate dehydrogenase A) blocks host-directed disruption of LCV by competitively binding the ASH (ASPM, SPD-2, Hydin) domain of OCRL (Oculocerebrorenal syndrome of Lowe), preventing Rab (rat sarcoma virus (Ras)-associated protein) GTP(guanosine triphosphate)ase-OCRL complex formation (145–148).

Lag-1 (O-acetyltransferase) is an accessory gene found in 80% of *L. pneumophila* clinical isolates and is involved in an intricate mechanism of host modulation in which it can recruit phagocytes through activation of the alternative complement pathway, which is incapable of clearing *L. pneumophila*, while also inhibiting the classical pathway, which does pose a threat to *L. pneumophila* (149). Activation of the alternative pathway benefits *L. pneumophila* by recruiting neutrophils that are also rendered incapable of phagolysosomal fusion by means of *lag-1* (149). Exogenous MOMP (major outer membrane protein) has been shown to facilitate chemotaxis via the complement system and inhibit phagocytosis in RAW264.7 cells, a mechanism necessary for both survival and host access (100). This broad repertoire of host-modulating proteins has allowed *Legionella* to survive and thrive in dangerous environments by hijacking hosts as a means of survival and nutrition.

Legionella's host modulation effects extend beyond evasion by being involved in nutrient uptake and disrupting other cellular processes. *L. pneumophila* has been known to express an atypical GTPase that disrupts host secretory pathways and induction of golgi fragmentation by hijacking host vesicular transport factor p115 through a mechanism distinct from eukaryote small GTPases (137). While golgi fragmentation has primarily been recorded in neurodegenerative diseases, it has been theorized to accelerate protein production by increasing surface area as a means to accelerate protein trafficking, processing, and sorting (150). This is among the ~180 putative GTPases encoded by *Legionella*, 104 of which being Ras-, Rho(Ras homolog)-, or Rab-GTPases which would be essential for survival within eukaryotic hosts (8).

There is also evidence of *Legionella* reprogramming host macrophage transcription through AnkH (Ankyrin H), an effector protein containing ankyrin repeats, which is traditionally used by eukaryote proteins to facilitate protein-protein interactions (131,151). Furthermore, *Legionella*'s VipA ((vacuolar protein sorting) VPS inhibitor protein A) and RidL effectors bind to actin and Vps29, respectively, as a method of

modulating host cell trafficking (152–154). This likely works in conjunction with several other secreted factors such as MavN (more regions allowing vacuolar colocalization N) to ensure that *Legionella* obtains sufficient nutrients such as iron (134).

1.8 Infection Models

Cell cultures are often maintained for periods of less than 2 months and are limited in their capacity to emulate multiple organ systems, thus complicating the natural simulation of a physiological T-cell response that would require time to dedicate to T-cell expansion (155). When studying secreted signals such as cytokines, the issues of media evaporation and waste buildup pose challenges in studying responses over 4-5 days.

In contrast, B-cells require less proliferation as B-cell-mediated immunity makes use of antibodies as their immunity-mediating agent, meaning that a relatively small population of cells can create enough antibodies but peak antibody counts require 2-3 weeks to achieve (156,157). However, cell culture would have a rather small diversity in their antibodies as each B-cell is only able to produce one Fab fragment and would not be able to properly facilitate the production or maturation required to allow for variations in Fab fragments to be produced (158). Progenitor B-cells that have not yet undergone gene splicing would be required to produce a more diverse population of Fab fragments. If B-cells used in culture do not produce antibodies that sufficiently recognize or neutralize the target antigen, these cells are rendered useless.

Common monocyte/macrophage cells used in research include bone marrow-derived macrophages (BMDM), peripheral blood mononuclear cells (PBMCs), THP-1, U937, and Mono-Mac-6 (MM6). Of these cells, BMDM are primary cells differentiated from mice bone marrow, which are mortal, and the other listed cells are immortalized cells from patients with leukemia. PBMCs are also primary cells from humans, but require donors and provide a mixed population of cells (2-10% of PBMCs are monocytes) (159–161). While primary cells are regarded as being more biologically relevant, they are more expensive and more difficult to obtain, having a limited lifespan, while cell lines are cheaper, offer more replicable results, and are immortal, albeit genetically less stable. In research settings all of the above cell types require the use of stimulating factors such as phorbol 12-myristate 13-acetate (PMA) and/or granulocyte/monocyte-colony stimulating factor (GM-CSF) (162). MM6 are more mature cells, THP-1 skews towards the M1 phenotype, and U937 skews towards the M2 phenotype (161,163). In terms of responses, phenotypically, THP-1 cells have been more responsive to stimulants such as IFN-γ and lipopolysaccharide, while RNAseq

data has suggested the opposite (161,164). Thus, these cells have some distinct properties setting them apart from each other.

Animal models offer a more complex system to simulate infection, coalescing a connected series of anatomical systems for a more comprehensive response. In many cases, animal models are the ideal preclinical model, however, this is not without controversy. Concerns have arisen regarding the replicability of animal models that mimic human diseases and their susceptibility to infection. This pushes researchers to consider affordability, practicality, and translatability when choosing models.

As the most widely used animal model, mice are cheap, easy to maintain, more customizable, have more available tools, and require smaller facilities. Unfortunately, mice have limited susceptibility to *Legionella* and the disease does not normally mimic that found in humans (165). Success has been found in using some strains of inbred mice, such as the A/J strain, to model infection at the cost of translatable immune expression, which have been noted to mimic outcomes seen in humans (165–167). Conversely, BALB/c and C57BL/6 mice, which are considered to be less permissive to *L. pneumophila* have been seen to be susceptible to *L. longbeachae*, likely owing to differences in localization within the lungs (167,168).

Guinea pigs have been recognized as experiencing infection pathophysiology more reminiscent of humans than mice, showing potential as an infection model, but are an uncommon model for immune responses (165,169,170). Where mice falter in their lack of susceptibility to *L. pneumophila* infections, guinea pigs are more susceptible than other rodent models displaying symptoms such as fever, impaired breathing, and weight loss (165,171). However, guinea pigs do possess pathogen recognition receptors analogous to those of humans, making them a potentially viable model for studying immune responses (172,173). Despite their drawbacks, both models have been used to simulate *Legionella* infections beyond the 5-day limit in cell culture, with infections of up to 10 days in guinea pigs and 7, 8, and even 21 days in A/J, CD1, and C57BL/6 mice, respectively, with the latter being infected with *L. longbeachae* (167,174,175). Thus, with the current state of animal models, guinea pigs are more suitable for studying pathology, but it is unclear how appropriate this model is for studying immunology. Furthermore, disseminated *Legionella* infections in animal models is a topic that remains sparse in the literature, which while uncommon, may occur in individuals with advanced LD or immune deficiencies (176–179). Lastly, the species under study should be considered when selecting a model, as some evidence suggests that while *L. pneumophila* has limited success in certain mouse models, *L. longbeachae* has been seen to be highly virulent in C57BL/6 mice (108).

1.9 Rationale

Research on *Legionella* infections has been largely limited to LPSG1. The focus on LPSG1 has been driven by epidemiology of LD infections and the dominance of LPSG1 diagnosed infections, albeit biased. For this reason, the inflammatory landscape during non-*pneumophila Legionella* infections is largely unknown.

1.10 Objectives and Hypothesis

To address the gaps in knowledge in *Legionella* diagnostics, **objective 1** was to explore the frequency of *Legionella* infections in individuals diagnosed with pneumonia and the performance of diagnostic techniques for detecting *Legionella* infections. The hypothesis was that LD caused by *L. pneumophila* is less common than what is commonly believed.

To answer the question of the etiology of LD and their detection rates, we conducted a scoping review to evaluate the efficacy of different diagnostic tests for *Legionella* and the detected species and serogroups.

To address these gaps in the field of *Legionella* infections, **objective 2** was to standardize intracellular growth of clinical isolates of *Legionella* and study the cytokine responses of macrophage-like U937 cells to infection by clinical isolates of *L. bozeman*ae, *L. dumoffii*, *L. micdadei*, *L. pneumophila*, and the American Type Culture Collection strain *L. pneumophila* Philadelphia-1. The hypothesis was that non-*pneumophila* species of *Legionella* will grow more slowly than *L. pneumophila* and induce a weaker cell mediated immune response. The immune response is expected to involve a mix of both proinflammatory and anti-inflammatory markers.

Chapter 2: The Adequacy of Current Legionnaires' Disease Diagnostic Practices in Capturing the Epidemiology of Clinically Relevant *Legionella*: A Scoping Review

2.1 Introduction

LD is caused by various species of *Legionella*, a genus of intracellular bacteria primarily found in water and soil. LD refers to pneumonia caused by *Legionella*. Legionellosis refers to *Legionella* infections, regardless of the site of infection. Within this text, Legionellosis is used when the source of the information uses the same terminology. Clinical symptoms range from mild febrile illness to severe and life-threatening pneumonia (24). *Legionella* is suspected to be underdiagnosed in community-acquired pneumonia (CAP) due to non-specific presenting symptoms and signs, and thus there is an under-recognition by treating clinicians (180). Pneumonia is commonly treated empirically without pathogen identification unless there is progressive clinical deterioration leading to further investigations (181). *Legionellae* are fastidious organisms that require special media for culture, and the sensitivity of culture techniques is low (182). These limitations in LD detection and diagnostics have resulted in up to 90% of LD diagnoses being missed (12).

In 2022, the World Health Organization reported the overall mortality by Legionnaires' disease as 5–10% and as high as 40–80% in immunosuppressed individuals (183). The incidence of disease is estimated to be ten to fifteen cases per million per year in Europe, Australia, and the USA (183). In 2019, the Public Health Agency of Canada stated that under 100 cases of LD were reported per year in Canada (184). However, provincial data suggest a higher number of cases within Canada, with Ontario reporting over 100 cases per year from 2013 to 2022 (10). Furthermore, LD cases have seen an increase across North America, as noted by the CDC and the British Columbia CDC (185,186).

Diagnostic testing for *Legionella* is rarely performed in mild CAP managed in the community or hospitalized individuals with mild to moderate pneumonia and is only undertaken in severely ill hospitalized patients once other avenues have been exhausted. However, despite guidelines recommending that patients requiring hospitalization for CAP be tested for *Legionella* among other pathogens that may not be responsive to empirical therapy, the narrow spectrum of applicability, the limitations of most *Legionella* diagnostics, and the previously low rate of testing often make this effort

fruitless (181,187,188). The CDC and the National Collaborating Centre for Infectious Disease (NCCID) recommend diagnostic testing for *Legionella* in outpatients failing antibiotic therapy, individuals requiring intensive care admission, immunocompromised individuals, individuals with recent travel history, or in the setting of a known Legionellosis outbreak (182,186,189). In addition, the CDC recommends testing for *Legionella* in cases of healthcare-associated pneumonia, and the NCCID recommends testing when there have been recent changes in water quality (189,190). A recent review indicated a 42% increase in LD in the 3 weeks following storms (191). LD is a looming hazard with the continuing rise in tropical storm intensity due to climate change, increased susceptibility associated with growing numbers of immunocompromised individuals, and a global aging population (191–193). Additionally, the shortening of winter and the increase in average winter temperatures extend periods of rain and warm weather, further contributing to the risk of LD (11,191,192,194,195).

Out of the 65 species of *Legionella*, only 25 are known to cause disease in humans (1). *L. pneumophila* is generally considered to be the most common cause of LD, followed by *L. longbeachae*, which is the cause of around 50% of LD cases in Oceania (196–198). All clinically relevant species of *Legionella* are primarily found in water except the soilborne *L. longbeachae*. Effective treatments for LD include fluoroquinolones and macrolides (199). Empirical therapy for CAP frequently includes either a macrolide or fluoroquinolones; however, when LD is not suspected and not treated, such as in immunocompromised people or people living with HIV, the outcomes and prognosis are adversely affected (83,200).

A summary of the diagnostic methods of LD is provided in Table 1. In most countries, the BinaxNOW UAT remains the primary diagnostic test, with a high sensitivity and quick turnaround time for only a single serogroup of *L. pneumophila* (serogroup 1) (189,190,201,202). In general, culture is the gold standard but has low sensitivity, requires invasive procedures to obtain samples (bronchoalveolar lavage) or involves samples that are infrequently produced in the context of LD (sputum), and offers no speciation in *Legionella* (38,39,189,203).

Table 1. Current *Legionella* diagnostic techniques outlined by the Centers for Disease Control and Prevention (CDC) (182) [a].

Test	Sensitivity (%)	Specificity (%)	Advantages	Disadvantages
Culture	20-80	100	Detects all species/serogroups	Technically difficult Slow (3-5 days to grow) Sensitivity dependent on technical skill Affected by appropriate antibiotic therapy No species identification without further testing
Urinary Antigen Test	70-100	95-100	Rapid Non-invasive	Only detects *L. pneumophila* serogroup 1 (LPSG1) Some do not excrete antigen or excrete the antigen intermittently
Polymerase Chain Reaction (PCR)	95-99	>99	Rapid Can detect species/serogroups other than LPSG1	Influenced by specimen quality Assays vary by laboratory Limited commercial availability
Direct Fluorescent Antibody	25-75	>95	Can detect species/serogroups other than LPSG1	Technically difficult Reagents may be difficult to obtain
Serology	80-90	>99	Can detect species/serogroups other than LPSG1	Antibodies may be shared across species/serogroups Cannot distinguish between current and past infection

[a] Modified from tables from the CDC (182).

The prevalence of *Legionella*, especially non-*pneumophila* and non-serogroup 1 *pneumophila*, is likely underreported due to infrequent sampling, the unavailability of diagnostic tools in medical facilities, difficulty collecting sputum, long turnaround times, and the fact that it is only studied in severe clinical presentations or an outbreak context (13,199,201). In this scoping review, we aimed to describe (1) the incidence, prevalence, and frequency of *Legionella* infections in individuals diagnosed with pneumonia with and without immunocompromised conditions, (2) the distribution of *Legionella* species and serotypes among people diagnosed with *Legionella*, and (3) which diagnostic techniques were used in each study.

2.2 Methods

2.2.1 Study type

We conducted a scoping review following the scoping review checklist to answer the following questions (204):

1. Does nucleic acid testing (NAT) increase the detection of non-*pneumophila* serogroup 1 *Legionella* compared to non-NAT?
2. Does immunocompromise increase the frequency of pneumonia caused by non-*pneumophila* serogroup 1 *Legionella* compared to non-immunocompromised individuals with LD?

The population, intervention/exposure, comparator, outcome, and timeframe (PICOT) table for our questions are shown below in table 2.

Table 2. PICOT table outlining our research questions

Question #	Population	Intervention/exposure (hypothesis)	Comparator	Outcome	Time frame
1	Individuals with pneumonia	Genotype-based techniques such as PCR, sequencing	Phenotype-based techniques such as culture, serology, DFA, and UAT	Incidence, prevalence, and frequency of *Legionella* compatibility (specific species/strains as stratified by molecular techniques)	N/A
2	Individuals with pneumonia who are immunocompro-mised	Genotype-based techniques such as PCR, sequencing	Phenotype-based techniques such as culture, serology, DFA, and UAT	Incidence, prevalence, and frequency of *Legionella* compatibility (specific species/strains as stratified by molecular techniques)	N/A

2.2.2 Search Strategy

We created a search strategy following PRISMA guidelines and searched in the following databases between 22 December 2022, and 12 February 2023: PubMed, PubMed Central, Cochrane Register of Controlled Trials, Clinicaltrials.org, and LegionellaDB. A timeframe was not included in the search. Our search queries, search strategies, and precise search dates can be found in Table 3. All searches were uploaded to Covidence, a web-based collaboration software platform that streamlines the production of systematic and other literature reviews (205).

Table 3. Search history, databases, and search terms, part 1 of 4. Boolean operators are bolded, and keywords are listed in brackets.

Date	Search Terms	Engine
December 21, 2022	((incidence) **OR** (epidemiology) **OR** (prevalence) **OR** (frequency)) **AND** ((diagnosis) **OR** (detection)) **AND** (legionella) **AND** ((pneumonia) **OR** (lung infection) **OR** (community acquired pneumonia) **OR** (coinfection) **OR** (lung abnormality) **OR** (lung abnormality) **OR** (lung abnormalities)) **AND** ((immunocompromised) **OR** (immunosuppressed) **OR** (HIV) **OR** (human immunodeficiency virus) **OR** (AIDS) **OR** (Acquired immunodeficiency syndrome) **OR** (cancer) **OR** (type 2 diabetes) **OR** (transplant) **OR** (autoimmunity) **OR** (arthritis) **OR** (IBD) **OR** (inflammatory bowel disease))	PubMed
December 21, 2022	((incidence) **OR** (epidemiology) **OR** (prevalence) **OR** (frequency)) **AND** ((diagnosis) **OR** (detection)) **AND** (legionella) **AND** ((pneumonia) **OR** (lung infection) **OR** (community acquired pneumonia) **OR** (coinfection) **OR** (lung abnormality) **OR** (lung abnormality) **OR** (lung abnormalities))	PubMed
December 21, 2022	((incidence) **OR** (epidemiology) **OR** (prevalence) **OR** (frequency)) **AND** ((diagnosis) **OR** (detection)) **AND** (legionella) **AND** ((pneumonia) **OR** (lung infection) **OR** (community acquired pneumonia) **OR** (coinfection) **OR** (lung abnormality) **OR** (lung abn**or**mality) **OR** (lung abnormalities)) **AND** ((immunocompromised) **OR** (immunosuppressed) **OR** (HIV) **OR** (human immunodeficiency virus) **OR** (AIDS) **OR** (Acquired immunodeficiency syndrome) **OR** (cancer) **OR** (type 2 diabetes) **OR** (transplant) **OR** (autoimmunity) **OR** (arthritis) **OR** (IBD) **OR** (inflammatory bowel disease))	Cochrane Register of Controlled Trials
December 21, 2022	((incidence) **OR** (epidemiology) **OR** (prevalence) **OR** (frequency)) **AND** ((diagnosis) **OR** (detection)) **AND** (legionella) **AND** ((pneumonia) **OR** (lung infection) **OR** (community acquired pneumonia) **OR** (coinfection) **OR** (lung abnormality) **OR** (lung abnormality) **OR** (lung abnormalities))	Cochrane Register of Controlled Trials
December 22, 2022	Legionnaires' disease	ClinicalTrials.gov

Boolean operators are bolded, and keywords are listed in brackets.

Continuation of Table 3, part 2 of 3

Date	Search Terms	Engine
December 22, 2022	(("epidemiology"[Subheading] **OR** "epidemiology"[All Fields] **OR** "incidence"[All Fields] **OR** "incidence"[MeSH Terms]) **OR** ("epidemiology"[Subheading] **OR** "epidemiology"[All Fields] **OR** "epidemiology"[MeSH Terms]) **OR** ("epidemiology"[Subheading] **OR** "epidemiology"[All Fields] **OR** "prevalence"[All Fields] **OR** "prevalence"[MeSH Terms]) **OR** ("epidemiology"[Subheading] **OR** "epidemiology"[All Fields] **OR** "frequency"[All Fields] **OR** "epidemiology"[MeSH Terms] **OR** "frequency"[All Fields])) **AND** (("diagnosis"[Subheading] **OR** "diagnosis"[All Fields] **OR** "diagnosis"[MeSH Terms]) **OR** detection[All Fields]) **AND** ("legionella"[MeSH Terms] **OR** "legionella"[All Fields]) **AND** (("pneumonia"[MeSH Terms] **OR** "pneumonia"[All Fields]) **OR** (("lung"[MeSH Terms] **OR** "lung"[All Fields]) **AND** ("infections"[MeSH Terms] **OR** "infections"[All Fields] **OR** "infection"[All Fields])) **OR** (("residence characteristics"[MeSH Terms] **OR** ("residence"[All Fields] **AND** "characteristics"[All Fields]) **OR** "residence characteristics"[All Fields] **OR** "community"[All Fields]) **AND** acquired[All Fields] **AND** ("pneumonia"[MeSH Terms] **OR** "pneumonia"[All Fields])) **OR** ("coinfection"[MeSH Terms] **OR** "coinfection"[All Fields]) **OR** (("lung"[MeSH Terms] **OR** "lung"[All Fields]) **AND** abnormality[All Fields]) **OR** (("lung"[MeSH Terms] **OR** "lung"[All Fields]) **AND** abnormality[All Fields]) **OR** (("lung"[MeSH Terms] **OR** "lung"[All Fields]) **AND** ("abnormalities"[Subheading] **OR** "abnormalities"[All Fields] **OR** "congenital abnormalities"[MeSH Terms] **OR** ("congenital"[All Fields] **AND** "abnormalities"[All Fields]) **OR** "congenital abnormalities"[All Fields]))) **AND** (("immunocompromised host"[MeSH Terms] **OR** ("immunocompromised"[All Fields] **AND** "host"[All Fields]) **OR** "immunocompromised host"[All Fields] **OR** "immunocompromised"[All Fields]) **OR** ("immunocompromised host"[MeSH Terms] **OR** ("immunocompromised"[All Fields] **AND** "host"[All Fields]) **OR** "immunocompromised host"[All Fields] **OR** "immunosuppressed"[All Fields]) **OR** ("hiv"[MeSH Terms] **OR** "hiv"[All Fields]) **OR** ("hiv"[MeSH Terms] **OR** "hiv"[All Fields] **OR** ("human"[All Fields] **AND** "immunodeficiency"[All Fields] **AND** "virus"[All Fields]) **OR** "human immunodeficiency virus"[All Fields]) **OR** ("acquired immunodeficiency syndrome"[MeSH Terms] **OR** ("acquired"[All Fields] **AND** "immunodeficiency"[All Fields] **AND** "syndrome"[All Fields]) **OR** "acquired immunodeficiency syndrome"[All Fields] **OR** "aids"[All Fields]) **OR** ("acquired immunodeficiency syndrome"[MeSH Terms] **OR** ("acquired"[All Fields] **AND** "immunodeficiency"[All Fields] **AND** "syndrome"[All Fields]) **OR** "acquired immunodeficiency syndrome"[All Fields]) **OR** ("neoplasms"[MeSH Terms] **OR** "neoplasms"[All Fields] **OR** "cancer"[All Fields]) **OR** ("diabetes mellitus, type 2"[MeSH Terms] **OR** "type 2 diabetes mellitus"[All Fields] **OR** "type 2 diabetes"[All Fields]) **OR** ("transplants"[MeSH Terms] **OR** "transplants"[All Fields] **OR** "transplant"[All Fields] **OR** "transplantation"[MeSH Terms] **OR** "transplantation"[All Fields]) **OR** ("autoimmunity"[MeSH Terms] **OR** "autoimmunity"[All Fields]) **OR** ("arthritis"[MeSH Terms] **OR** "arthritis"[All Fields]) **OR** IBD[All Fields] **OR** ("inflammatory bowel diseases"[MeSH Terms] **OR** ("inflammatory"[All Fields] **AND** "bowel"[All Fields] **AND** "diseases"[All Fields]) **OR** "inflammatory bowel diseases"[All Fields] **OR** ("inflammatory"[All Fields] **AND** "bowel"[All Fields] **AND** "disease"[All Fields]) **OR** "inflammatory bowel disease"[All Fields]))	PubMed Central

Boolean operators are bolded, and keywords are listed in brackets.

Continuation of Table 3, part 3 of 3

Date	Search Terms	Engine
December 22, 2022	(("epidemiology"[Subheading] **OR** "epidemiology"[All Fields] **OR** "incidence"[All Fields] **OR** "incidence"[MeSH Terms]) **OR** ("epidemiology"[Subheading] **OR** "epidemiology"[All Fields] **OR** "epidemiology"[MeSH Terms]) **OR** ("epidemiology"[Subheading] **OR** "epidemiology"[All Fields] **OR** "prevalence"[All Fields] **OR** "prevalence"[MeSH Terms]) **OR** ("epidemiology"[Subheading] **OR** "epidemiology"[All Fields] **OR** "frequency"[All Fields] **OR** "epidemiology"[MeSH Terms] **OR** "frequency"[All Fields])) **AND** (("diagnosis"[Subheading] **OR** "diagnosis"[All Fields] **OR** "diagnosis"[MeSH Terms]) **OR** detection[All Fields]) **AND** ("legionella"[MeSH Terms] **OR** "legionella"[All Fields]) **AND** (("pneumonia"[MeSH Terms] **OR** "pneumonia"[All Fields]) **OR** (("lung"[MeSH Terms] **OR** "lung"[All Fields]) **AND** ("infections"[MeSH Terms] **OR** "infections"[All Fields] **OR** "infection"[All Fields])) **OR** (("residence characteristics"[MeSH Terms] **OR** ("residence"[All Fields] **AND** "characteristics"[All Fields]) **OR** "residence characteristics"[All Fields] **OR** "community"[All Fields]) **AND** acquired[All Fields] **AND** ("pneumonia"[MeSH Terms] **OR** "pneumonia"[All Fields])) **OR** ("coinfection"[MeSH Terms] **OR** "coinfection"[All Fields]) **OR** (("lung"[MeSH Terms] **OR** "lung"[All Fields]) **AND** abnormality[All Fields]) **OR** (("lung"[MeSH Terms] **OR** "lung"[All Fields]) **AND** abnormality[All Fields]) **OR** (("lung"[MeSH Terms] **OR** "lung"[All Fields]) **AND** ("abnormalities"[Subheading] **OR** "abnormalities"[All Fields] **OR** "congenital abnormalities"[MeSH Terms] **OR** ("congenital"[All Fields] **AND** "abnormalities"[All Fields]) **OR** "congenital abnormalities"[All Fields]))))	PubMed Central
January 5, 2023	((incidence) **OR** (distribution) **OR** (epidemiology) **OR** (prevalence) **OR** (frequency) **OR** (specificity) **OR** (sensitivity) **OR** (etiology)) **AND** ((diagnosis) **OR** (detection)) **AND** (legionella) **AND** ((pneumonia) **OR** (lung infection) **OR** (community acquired pneumonia) **OR** (coinfection) **OR** (lung abnormality) **OR** (lung abnormalities))	PubMed
February 10, 2023	((incidence) **OR** (distribution) **OR** (epidemiology) **OR** (prevalence) **OR** (frequency) **OR** (specificity) **OR** (sensitivity) **OR** (etiology)) **AND** ((diagnosis) **OR** (detection)) **AND** (legionella) **AND** ((pneumonia) **OR** (lung infection) **OR** (community acquired pneumonia) **OR** (coinfection) **OR** (lung abnormality) **OR** (lung abnormalities) **OR** (Legionnaires disease) **OR** (Legionellosis) **OR** (Pontiac fever) **OR** (pneumonitis) **OR** (bronchopneumonia))	PubMed
February 10, 2023	Papers outlined in LegionellaDB that either listed identification of a non-pneumophila serogroup 1, Legionella spp., or Legionella pneumophila. Did not include Unknown serogroups/species	LegionellaDB

Boolean operators are bolded, and keywords are listed in brackets.

2.2.3 Study Selection

We included studies that met all of the following criteria: (1) original research that reports data about the PICOT questions that can be used to calculate incidence, frequency, or prevalence of *Legionella* with a species or serogroup analysis; (2) comparative quantitation using multiple detection methods; (3) use of at least one NAT- and one non-NAT-based technique for diagnosis; (4) at least 5 cases of LD in the patient group. During the title and abstract screening, articles were included if there was an indication of clinical diagnosis of *Legionella*.

We excluded studies with the following criteria: (1) case reports or series of <5 patients; (2) studies missing serogroup or species analysis; (3) studies in which patients were only infected with *L. pneumophila* serogroup 1; (4) articles not available in English; (5) articles with no abstract; (6) articles examining environmental distribution; (7) ongoing trials; (8) studies not conducted on humans or using human samples; (9) non-pneumonic Legionellosis; (10) articles in which the diagnostic techniques are not reported.

Titles and abstracts were screened independently by two blinded reviewers using Covidence (205). References that met all the inclusion criteria without exclusions were selected for full-text review and data extraction. Discrepancies were resolved by consensus or with a third reviewer where necessary.

2.2.4 Data Extraction

The following data were extracted from the included studies: (1) year of study; (2) country(ies) or continent where the study was conducted; (3) population under study; (4) sample size disaggregated by sex; (5) diagnostic techniques used; (6) brand of tests, if listed; (7) species or serogroups found; (8) immunosuppressive conditions; (9) comorbidities; (10) time of follow-up, if listed; (11) limitations, both recognized and unrecognized by original authors.

2.2.5 Data Synthesis

Descriptive analyses were conducted to report the frequency at which each serogroup or species was found clinically and the diagnostic testing used. Some studies were only eligible to describe the *Legionella* subgroup (i.e., species and/or serogroups) breakdown within a population, while other studies allowed for an analysis of the specific by-technique breakdown for each subgroup. Studies fulfilling the latter criterion were used to conduct an analysis on the testing positivity of different techniques. Furthermore, articles providing LD cases within a greater pneumonia context were subject to an analysis on their frequency within this context.

2.3 Results

Of the 3449 article citations identified, we included 279 unique studies for full-text review and 31 in the analysis (Figure 1). Many excluded articles were excluded for multiple reasons. However, we can only list one reason in Covidence. Table 4 reports all the included articles, regions, populations studied, sample sizes, diagnostic tests used, and outcomes.

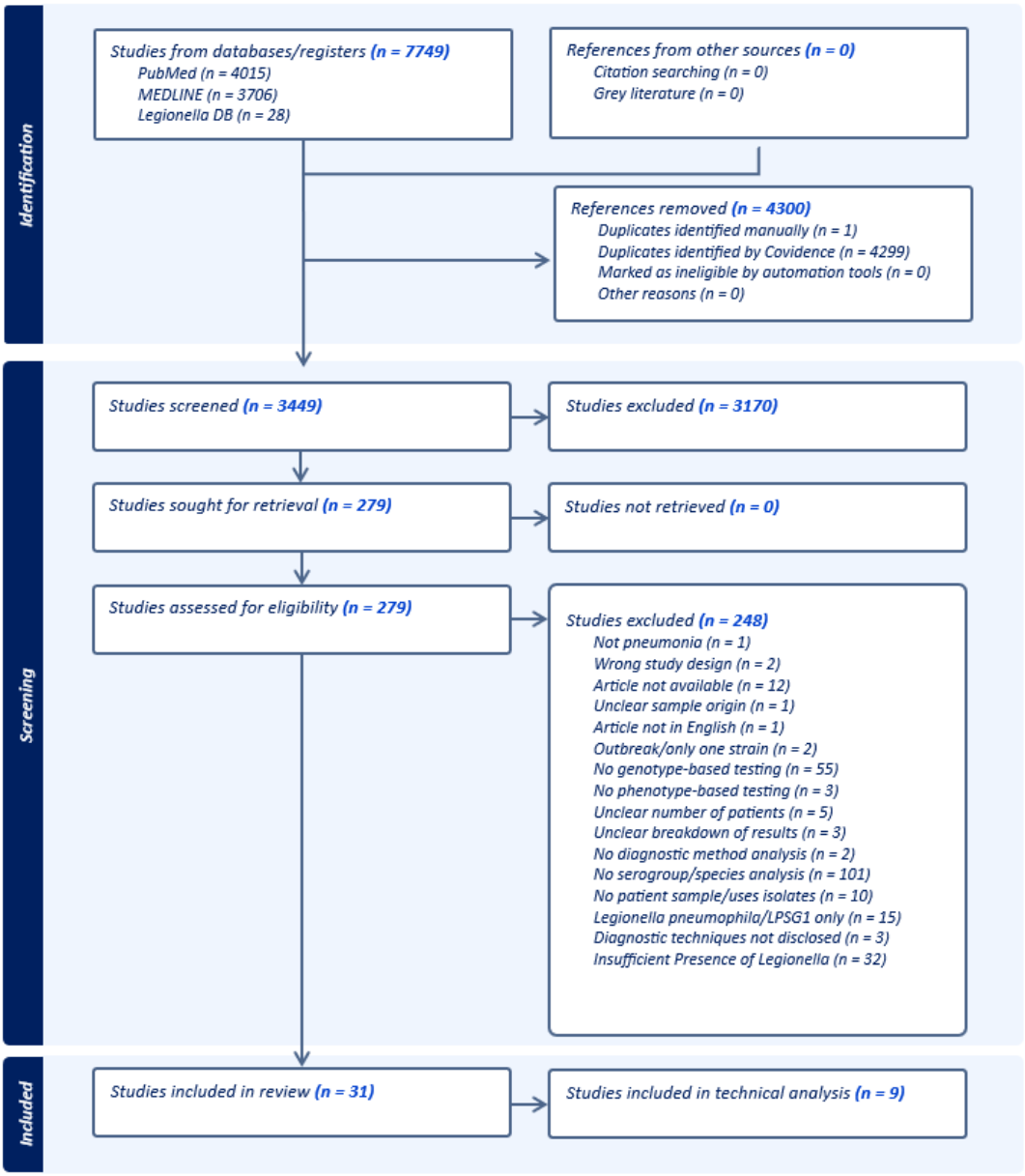


Figure 1. PRISMA flowchart depicting the screening process generated by Covidence (205).

Table 4. Summary of studies included in the scoping review, part 1 of 3.

First Author, year of publication, reference	Region(s)	Year(s) of study	Population	Sample size (cases)	Sample types	Techniques used	*Legionella* species or serogroups found [b]
Alexiou-Daniel, et al., 1998, (206)	Greece	1993-1998	Hospitalized legionellosis patients	24	Serum	Serology [a], culture, PCR	22 LPSG1, 2 LpSG4
Beauté, 2017, (207)	European Union states, Iceland, Norway	2011-2015	*Legionella*-infected individuals	30532	Urine, serum	UAT, culture, PCR	3020 LPSG1, 19 LpSG2, 101 LpSG3, 13 LpSG4, 19LpSG5, 42 LpSG6, 9 LpSG7, 8 LpSG8, 5 LpSG9, 19 LPSG10, 3 LPSG11, 1 LPSG12, 2 LPSG13, 7 LPSG14, 4 mixed SG, 7 non-LPSG1, 232 Lp unknown SG, 2 La, 15 Lb, 1 Lci, 2 Ldu, 35 Ll, 1 Lma, 12 Lmi, 1 Ls, 27 other *Legionella* species, 38 unknown *Legionella* species
Berger et al., 2006, (208)	France	2002-2003	ICU pneumonia	210	BALF, serum, urine	RT-PCR, UAT, serology, culture	10 Lp, 3 Lb, 2 La, 2 Lr, 1 Lq, 1 Lwo
Cameron, 2016, (209)	Scotland	2008-2014	*Legionella*-infected individuals	37	Respiratory samples, sputum, urine	PCR, serology, culture, UAT	12 Ll, 25 Lp
Diederen, et al., 2008, (210)	Netherlands	1998-2000	CAP adults	242	Sputum, endotracheal aspirates, lung biopsy, bronchoscopic specimens	RT-PCR, UAT, blood culture	11Lp, 2 non-*pneumophila Legionella*
Diederen, et al., 2009, (211)	Netherlands	2002-2005	Pneumonia compatibility	151	Sputum, blood, urine, serum, BALF	RT-PCR, culture, UAT, ELISA	36 Lp, 4 non-*pneumophila Legionella*
Elverdal et al., 2013, (212)	Denmark	2008-2010	Pneumonia (hospitalized)	10503	Serum, LRT samples, blood, urine	ELISA, culture, UAT, PCR	35 LPSG1, 1 LpSG2, 11 LpSG3, 1 LpSG5, 3 LpSG6, 62 *Legionella* spp.
Ghorbani et al., 2021, (213)	Iran	2019-2020	Pneumonia (hospitalized)	123	Sputum, BALF, pleural aspirates	Culture, RT-PCR	8 Lp, 1 Lch
Isenman et al., 2016, (16)	New Zealand	2009-2013	*Legionella*-infected individuals	126	Urine, LRT samples, serum	Culture, PCR, UAT, serology	107 Ll, 19 Lp
Jespersen et al., 2009, (214)	Denmark	1995-2005	*Legionella*-infected individuals	370	Urine, serum	UAT, serology, PCR, culture	110 LPSG1, 4 LpSG2, 39 LpSG3, 3 LpSG4, 6 LpSG6, 4 Lp unknown serogroup, 4 Lb, 2 Lmi, 161 unknown *Legionella* species
de Jong et al., 2010, (195)	Europe	2010	*Legionella*-infected travelers	864	Urine, serum	UAT, PCR, culture, serology	672 LPSG1, 3 LpSG3, 2 LpSG6, 1 LPSG12, 3 mixed SG, 158 unknown SG Lp, 1 Lb, 10 unknown species, 14 unreported species
Joseph, 2004, (215)	Europe	2000-2002	*Legionella*-infected travelers	10322	Urine, serum	UAT, serology, PCR, direct antigen, culture	7900 LPSG1, 1749 Lp, 9 LpSG2, 35 LpSG3, 5 LpSG4, 10 LpSG5, 22 LpSG6, 1 LpSG7, 2 LpSG8, 7 LPSG10/14, 673 non-*pneumophila Legionella*, 2 La, 4 Lb, 2 Ldu, 1 Lg, 3 Ll

Continuation of table 4, part 2 of 3.

First Author, year of publication, reference	Region(s)	Year(s) of study	Population	Sample size (cases)	Sample types	Techniques used	*Legionella* species or serogroups found [b]
Joseph et al., 2010, (216)	Europe	2007-2008	*Legionella*-infected individuals	11867	Serum, urine, respiratory samples,	Culture, UAT, serology, respiratory antigen, PCR	9436 LPSG1, 1785 non-SG1 Lp, 646 unknown/other species
Kim et al., 2015, (217)	South Korea	2000-2001	Suspected LD	10	Sputum, urine	UAT, serology, RT-PCR, culture	5 LPSG1, 1 LpSG 2-14, 4 unknown *Legionella* spp.
Lever, 2003, (218)	Europe	2000-2001	*Legionella*-infected travelers	841	Urine, serum, respiratory samples	UAT, serology, PCR, culture	303 LPSG1, 407 other serogroup/species
Lindsay et al., 1994, (219)	Scotland	N/A	Proven cases of LD	5	Serum, urine	UAT, serology, culture, PCR	4 LPSG1, 1 LPSG12
Löf et al., 2021 (220)	Sweden	2018	non-*pneumophila Legionella* cases	41	N/A	UAT, RT-PCR, culture, serology	6 non-*pneumophila Legionella*, 33 Ll, 2 Lb
Maniwa et al., 2006, (221)	Japan	1999-2005	*Legionella*-infected individuals	30	Urine, sputum, BALF, serum	culture, UAT, PCR, serology	10 LPSG1, 2 LpSG6, 1 Ll, 17 unknown *Legionella* species
Murdoch et al., 1996, (19)	New Zealand	1992-1995	Previously confirmed LD positive or negative	52	Urine, serum	PCR, culture, serology, ELISA	2 LPSG1, 1 LpSG3, 3 LpSG4, 2 LpSG5, 1 LpSG6, 1 LpSG7, 1 LPSG10, 2 LPSG12, 2 LPSG13, 10 Lmi, 3 Ll, 3Lj, 1 Lb, 1 Lg
Pasculle et al., 1989, (222)	US	1987	*Legionella*-infected individuals	809	Sputum	ELISA, culture, DNA probe	6 LPSG1, 2 LpSG4, 1 LpSG6, 1 Lmi, 2 LPSG1/Lmi
Pouderoux et al., 2020, (223)	France	2013-2017	Culture positive LD	1686	Sputum, bronchial aspirate, BALF	Culture, real-time RT-PCR, WGS, serology	9 LPSG1, 1 LpSG3, 1 LpSG8, 1 *Legionella* spp., 1 LpSG2/6/12
Priest et al., 2019, (17)	New Zealand	2015-2016	Pneumonia	4826	LRT specimens, urine	PCR, culture, MALDI-TOF, serology, UAT	52 Lp, 150 Ll, 24 other *Legionella* species, 12 non-speciated *Legionella*
Qin et al., 2016, (224)	China	2012-2013	Pneumonia or LRTIs in hospital	624	BAL, sputum	Culture, RT-PCR, sequencing	70 Lp, other *Legionella* species 1
Ricketts et al., 2005, (225)	Europe	2003-2004	*Legionella*-infected individuals	9166	Urine, serum, respiratory samples	Culture, UAT, serology, antigen detection, PCR	7007 LPSG1, 1526 non-SG1 Lp, 633 other *Legionella* spp
Ricketts et al., 2007, (226)	Europe	2005-2006	*Legionella*-infected individuals	11980	Respiratory samples, urine, serum	Culture, UAT, serology, PCR	9219 LPSG1, 1862 Lp non-SG1/unknown SG, 899 unknown *Legionella* species

Continuation of table 4, part 3 of 3.

First Author, year of publication, reference	Region(s)	Year(s) of study	Population	Sample size (cases)	Sample types	Techniques used	*Legionella* species or serogroups found [b]
Ricketts et al., 2010, (216)	Europe	2008	*Legionella*-infected travelers	866	N/A	Culture, serology, PCR, UAT	57 LPSG1, 1 LpSG2, 1 LpSG3, 3 Lp unknown SG, 1 unknown *Legionella* spp
Scaturro et al., 2021, (227)	Italy	2018	Pneumonia patients	33	Urine, serum, respiratory secretions	UAT, RT-PCR, culture, serology, single high titer	18 Lp unclear SG, 15 LpSG2
Sivagnanam et al., 2017, (228)	US	1999-2013	Transplant recipients suspected of *Legionella* infection	4090	BAL, blood, sputum, urine, tissue	Culture, UAT, sequencing, MALDI-TOF	7 LPSG1, 8 unknown SG Lp, 10 Lmi, 4 Ll, 1 Lwa, 1 Lt, 1 Ldu
Tateda et al., 1998, (229)	Japan	N/A	Suspected *Legionella* infection	36	Sputum, BAL, serum, urine	Culture, serology, UAT, PCR	12 Lp, 1 Lb, 1Lp/Ldu
Waller et al., 2022, (196)	Australia	2010-2021	Legionellosis patients	53	Urine, serum	UAT, serology, PCR	31 Ll, 22 Lp, 2 *Legionella* spp.

ELISA includes DFA. Unspecified PCR techniques are listed as "PCR". Abbreviations: LP, *L. pneumophila*; Lb, *L. bozemanii* or *L. bozemanae* (sic); La, *L. anisa*; Lr, *L. rubrilucens*; Lq, *L. quinlivanii*; Lwo, *L. worsleiensis*; Lci, *L. cincinnatiensis*; Ll, *L. longbeachae*; SG, serogroup; Ldu, *L. dumoffii*; Lmi, *L. micdadei*; Lj*, L. jordanis*; Lg, *L. gormanii*; Lwa, *L. wadsworthii*; Lt, *L. tucsonensis*; Lch, *L. cherrii*; Lma, *L. maceachernii*; Ls, *L. sainthelensi*; CAP, community-acquired pneumonia; BALF, bronchoalveolar lavage fluid; LRT, lower respiratory tract; PCR, polymerase chain reaction; UAT, urinary antigen test; ELISA, enzyme-linked immunosorbent assay; WGS, whole-genome sequencing; SBT, sequence-based typing; RT, real-time. [a] Serology includes seroconversion and high antibody titers against *Legionella*. [b] Species separated by a slash ("/") are present as coinfections.

When looking strictly at studies reporting cases of pneumonia, regardless of pathogen identification, the majority of LD cases were found to be caused by *L. pneumophila* that was not serogrouped followed by *L. longbeachae* and unidentified species of *Legionella* depending on the population included, with other identified species and serogroups being few and far between (Tables 5 and 6). Relative to the total pneumonia cases, the most prevalent subgroups of *Legionella* are *L. pneumophila*, *L. longbeachae*, and unknown species of *Legionella* appearing in 1.4% (summed up across all serogroups), 0.895%, and 0.627% of the population, respectively (Table 5). However, a significant portion of these were due to *L. pneumophila* of unknown serogroup (1.140%) and unknown *Legionella* species (0.627%). For many subgroups, there is a wide range in their frequency of diagnosis.

Relative representation of subgroups relative to total reported LD cases is as follows: *Legionella pneumophila* serogroup 1, *L. pneumophila* of an undetermined serogroup, unspeciated *Legionella*, and *L. longbeachae* are the most common at 50.064%, 9.816%, 38.862%, and 0.482%, respectively (Table 6).

In studies involving national surveillance, the total population is reduced to either that of individuals diagnosed with pneumonia or LD, depending on the information provided in the study. Subgroups examined in only one eligible study have their frequencies marked with an asterisk (*) (Tables 5 and 6).

Table 5. Breakdown of reported cases of *Legionella* subgroups relative to the cumulative population of pneumonia cases [a].

Species	People diagnosed with Legionella	Total population diagnosed with pneumonia	Frequency	Frequency range
LPSG1	35	16751	0.209%	0.333%-23.8%
LpSG2	1		0.006%	0.0095%*
LpSG3	11		0.066%	0.105%*
LpSG5	1		0.006%	0.0095%*
LpSG6	3		0.018%	0.00286%*
Lp unknown SG	199		1.190%	1.07%-33.3%
L. longbeachae	150		0.895%	0.0978%-3.085%
L. bozemanae	4		0.024%	1.43%-2.778%
L. anisa	2		0.012%	0.952%*
L. rubrilucens	2		0.012%	0.952%*
L. cherrii	1		0.006%	0.813%*
L. dumoffii	1		0.006%	2.778%*
L. quinlivanii	1		0.006%	0.476%*
L. worsleiensis	1		0.006%	0.476%*
Unknown *Legionella* spp.	105		0.627%	0.160%-2.649%
TOTAL	**509**		**3.086%**	-

[a] Data compiled from 9 studies. Abbreviation(s): Lp, *L. pneumophila*; SG, serogroup.[a] In studies involving national surveillance, the total population is reduced to that of individuals diagnosed with pneumonia based on the information provided in the study. Studies only reporting patients who were positive for Legionnaires' disease were excluded from the analysis summarized in this table. Total population includes individuals who presented with pneumonia but tested negative for *Legionella* and those who tested positive. Frequency ranges show the frequency of *Legionella* within the population of a given study. Serogroups and species examined in only one eligible study for this analysis have their frequencies marked with an asterisk (*).

Table 6. Relative frequency of *Legionella* serogroups and species compared to total Legionella-positive cases in the literature [a].

Serogroups	# of Cases	Frequency
LPSG1	38506	50.064%
Lp Unknown serogroup	7550	9.816%
LpSG3	194	0.252%
LpSG6	79	0.103%
LpSG2	51	0.066%
LpSG5	32	0.042%
LpSG4	28	0.036%
LPSG10	27	0.035%
LPSG14	14	0.018%
LpSG7	11	0.014%
LpSG8	11	0.014%
LpSG9	7	0.008%
LPSG12	6	0.008%
LPSG13	4	0.005%
LPSG11	3*	0.004%
Species		
Unknown *Legionella* spp.	29890	38.862%
L. longbeachae	371	0.482%
L. micdadei	37	0.048%
Unknown non-*pneumophila Legionella*	37	0.048%
L. bozemanae	31	0.040%
L. anisa	6	0.008%
L. dumoffi	6	0.008%
L. gormanii	3	0.004%
L. maceachernii	2	0.003%
L. rubrilucens	2*	0.003%
L. quinlivanii	1*	0.001%
L. worsleiensis	1*	0.001%
L. cherrii	1*	0.001%
L. wadsworthii	1*	0.001%
L. tucsonensis	1*	0.001%
L. cincinnatiensis	1*	0.001%
L. sainthelensi	1*	0.001%

[a] Serogroups and species examined in only one eligible study have their frequencies marked with an asterisk (*). Abbreviations: Lp, *L. pneumophila*; SG, serogroup. Data compiled from 31 studies.

Seven studies qualified for our technical analysis, which required breakdown by technique, including the reporting of all techniques performed on each sample instead of only listing the main technique used for diagnosis (Table 7). Additionally, studies were unclear about whether testing using the UAT was attempted for initial diagnoses of cases later found to be compatible with non-pneumophila serogroup 1 *Legionella* but not LPSG1. Where it would normally be expected to give a negative result, it is unclear if the UAT was used at all, as the studies did not report its use either way.

Table 7. Qualitative summary of diagnostics by *Legionella* species and serogroup, separated by technique, part 1 of 2 [a].

First Author	Spp/SG	UAT	PCR	Serology	DFA	Culture	DNA probe
Ghorbani (213)	*L. pneumophila*	-	7/7	-	-	2/7	-
	L. cherrii	-	1/1	-	-	0/1	-
	Total	**-**	**8/8**	**-**	**-**	**2/8**	**-**
Isenman (16)	*L. longbeachae*	2/99	107/107	10/10	-	44/107	-
	L. pneumophila	-	19/19	-	-	12/19	-
	Total	2/99	**126/126**	**10/10**	**-**	**56/126**	**-**
Lindsay (219)	LPSG1	4/4	4/4	4/4	-	1/3	-
	LPSG12	0/1	1/1	1/1	-	0/1	-
	Total	**4/5**	**5/5**	**5/5**	**-**	**1/4**	**-**
Murdoch (19)	LPSG1	-	Serum:0/2 Urine:1/2	-	1/2	2/2	-
	LPSG3	-	Serum:0/1 Urine:0/1	-	0/1	0/1	-
	LPSG4	-	Serum:1/2 Urine:2/2	-	0/1	0/1	-
	LPSG4/5	-	Serum:1/1 Urine:1/1	-	1/1	1/1	-
	LPSG5	-	Serum:1/1 Urine:0/1	-	1/1	0/1	-
	LPSG6	-	Serum:0/1 Urine:0/1	-	0/1	0/1	-
	LPSG7	-	Serum:1/1 Urine:1/1	-	1/1	1/1	-
	LPSG10/12	-	Serum:0/1 Urine:1/1	-	0/1	0/1	-
	LPSG12/13	-	Serum:0/1 Urine:0/1	-	0/1	0/1	-
	LPSG13	-	Serum:0/1 Urine:1/1	-	1/1	1/1	-
	**L. micdadei*	-	Serum:4/10 Urine:5/10	-	4/6	0/6	-
	**L. longbeachae*	-	Serum:1/3 Urine:0/3	-	2/2	0/2	-
	***L. jordanis*	-	Serum:3/3 Urine:0/3	-	0/3	0/3	-
	***L. bozemanae*	-	Serum:1/1 Urine:0/1	-	0/1	0/1	-
	L. gormanii	-	Serum:0/1 Urine:1/1	-	-	-	-
	Total	**-**	**Serum:13/30 Urine:13/30**	**-**	**11/23**	**5/23**	**-**
Pasculle (Admission) (222)	LPSG1	-	-	-	5/8	8/8	6/8
	LPSG4	-	-	-	2/2	2/2	2/2
	LPSG6	-	-	-	1/1	1/1	1/1
	L. micdadei	-	-	-	3/3	3/3	3/3
	Total	**-**	**-**	**-**	**11/14**	**14/14**	**12/14**
Pasculle (Follow-up)(222)	LPSG1	-	-	-	8/8	8/8	8/8
	LPSG4	-	-	-	2/2	2/2	2/2
	LPSG6	-	-	-	1/1	1/1	1/1
	L. micdadei	-	-	-	3/3	3/3	3/3
	Total	-	-	-	14/14	14/14	14/14

Continuation of table 7, part 2 of 2.

First Author	Spp/SG	UAT	PCR	Serology	DFA	Culture	DNA probe
Pouderoux (Initial infection) (223)	LPSG1	9/10	-	-	-	9/10	-
	LPSG3	-	-	-	-	1/1	-
	LPSG8	-	-	-	-	1/1	-
	Total	**9/10**	**-**	**-**	**-**	**11/12**	**-**
Pouderoux (Recurrent infection) (223)	LPSG1	-	7/10	-	-	8/10	-
	LPSG3	-	1/1	-	-	1/1	-
	LPSG2-6-12	-	1/1	-	-	1/1	-
	Total	**-**	**9/12**	**-**	**-**	**10/12**	**-**
Waller (196)	*L. pneumophila*	8/16	4/5	Acute:10/16 Convalescent:2/11	-	-	-
	L. longbeachae	-	3/6	Acute:19/28 Convalescent:9/30	-	-	-
	Total	**8/16**	**7/11**	**Acute:29/44 Convalescent:11/41**	**-**	**-**	**-**
Cumulative Total	**-**	**23/130**	**181/222**	**55/100**	**36/51**	**113/213**	**26/28**
Frequency of positivity (%)	**-**	**17.69**	**81.53**	**55.00**	**70.59**	**53.05**	**92.86**

*These species were observed in a coinfection. ** These species were observed in a separate coinfection. [a]All cases outlined in this table are confirmed cases. Numerators are the number of positive tests, while denominators are the number of total tests applied to that species or serogroup. Pasculle et al. included data from initial diagnoses and repeat diagnostics conducted over 9 days of patient hospitalization (222). Results from both datasets are listed separately. Slowly resolving Legionnaires' disease cases from Pouderoux et al. are included in the initial infections, while reinfections/recurrent infections are outlined in recurrent infections (223). Abbreviations: SG, serogroup; UAT, urinary antigen test; PCR, polymerase chain reaction; DFA, direct fluorescent antibody.

2.4 Discussion

Our review found that most LD cases are caused by an unidentified species or serogroup of *L. pneumophila*. The emphasis on using a UAT that strictly detects LPSG1 as an initial test likely results in a significant number of missed cases (13,230,231). Typically, culture-positive diagnoses are not subject to speciation or serogrouping or the species could not be identified for other reasons (207). We found that almost 50% of LD cases are still caused by an unspecified species or a serogroup not detected by the standard UAT (Table 6). In general, the detection of the respiratory pathogen causing CAP is very low. Jain et al. found that only 38% of individuals with CAP were positive for pathogen detection (14).

The continued belief that LD is almost exclusively caused by *L. pneumophila* SG1 and the accompanying diagnostic practices leads to further missed diagnoses and increasingly discordant epidemiological data. A broad diagnostic coverage of *Legionella* is crucial, as the disease has been reported to be similar in both manifestations and outcomes across subgroups (209,232,233). LD diagnosis is very narrow in scope and species identification is often not involved, further reinforcing the small pool of clinically relevant *Legionella*. While the BinaxNOW Legionella UAT has a rapid turnaround time, it is strictly capable of detecting *L. pneumophila* serogroup 1. The RIBOTEST *Legionella*, another UAT, is used exclusively in Japan and can detect *Legionella pneumophila* serogroups 1–15 and several other species at a sensitivity comparable to BinaxNOW *Legionella*'s rate of detection for LPSG1 strains suspended in saline solutions (234). The other first-line diagnostic is culture, which is hampered by low sensitivity, various cysteine-containing media with advantages and disadvantages, takes several days, and has variable performance between laboratories. One potential hurdle to culture usage beyond the slow growth rate of *Legionella* species is the use of BCYE media without antibiotics, allowing the growth of other bacteria. Some newer products have been released on the market that may improve the landscape of *Legionella* diagnostics. Among these is the BioFire® FilmArray® Pneumonia (PN) Panel, which can detect up to 33 bacterial and viral targets (including *Legionella pneumophila*) in bronchoalveolar lavage fluid or sputum (235).

Based on our dataset, we found that NAT-based techniques had a higher frequency of *Legionella* positivity compared to non-NAT-based ones (Table 7). PCR remains unstandardized across institutions, as many groups use in-house primers and divergent protocols. Ideally, PCR should capture a diverse population of *Legionella* by using a multiplex panel of primers targeting genus-conserved sequences (e.g., 16S rRNA) and species-conserved sequences in some of the more common species of *Legionella* such as *L. pneumophila* and *L. longbeachae*, with some adjustments made based on locality. While costly, the adoption of sequencing into diagnostics would contribute to better surveillance of clinically relevant

strains, changes in drug resistance, and mutations over time (236). Of note, European countries have shifted towards sequence-based typing for *Legionella* rather than serogroup, which can be used to obtain sequences and typify from culture-negative samples (237).

One of the key issues in pneumonia treatment is that the etiology of CAP is rarely identified, and empirical treatments may not cover atypical bacteria, especially within the context of PLHIV (181,238). In most scenarios, the American Thoracic Society and Infectious Diseases Society of America do not recommend microbiological sampling to ascertain etiological diagnosis until initial therapies have been exhausted, even suggesting the aversion of the UAT, unless the disease is severe or resistant organisms are suspected (181,239). *Legionella* cases requiring intensive care unit admissions are associated with delayed urinary antigen testing and presumably *Legionella* testing in general (64). Conversely, early concordant treatment reduces the probability of intensive care unit admission (64,83). Li et al. found that next-generation sequencing effectively detected fastidious organisms including *Legionella* in cases that culture failed to identify, even detecting pathogens when culture test results were negative, resulting in adjusted treatment in 55% of patients (84). Thus, the delays and mistakes in *Legionella* identification are likely contributing to the high number of cases requiring hospitalization and intensive care.

Presumably, the reported case numbers of non-LPSG1 represent underestimates due to missed diagnoses. This is a conceivable scenario, as there are regional variations in species distribution and unidentified species were the third most common finding in our review (240). Furthermore, many studies use culture or UAT for the initial diagnosis before further identification, only testing samples with other techniques when initial tests yield positive results. Necessitating a positive result from these techniques introduces a bias toward the detected species or serogroups. Samples for culture are rare due to difficulties in obtaining bronchoalveolar lavage and sputum, while the UAT is over-selective (13,84,190,201). Our findings show that the subgroups that warrant more deliberate monitoring are *L. pneumophila* regardless of serogroup, *L. longbeachae*, *L. micdadei*, and *L. bozemanae* (Tables 5 and 6).

While it has been widely recognized that diagnosis of *Legionella* pneumonia is poor, table 7 highlights the need for diagnostics to be more comprehensive. While accommodating the detection of all species of *Legionella*, the inter-study and presumably inter-lab consistency of PCR is unknown. The information included in our dataset suggests that PCR and DNA probes had a higher frequency of positivity, regardless of *Legionella* subgroup (Table 7). Of note, DNA probes were only used in one study, and of the studies that reported which type of PCR was used, all used real-time PCR. The difficulty in recovering respiratory samples further encourages the merits of testing multiple specimens. Without having to develop new

techniques or products to detect *Legionella* in patient samples, there is a benefit in adopting a more comprehensive diagnostic regimen as conducted by Pasculle et al. and Decker et al. (64,199,222,241). However, these are not without the drawback of a higher rate of false positives.

Beyond the consequences of a delayed diagnosis and impeded timely concordant treatment, limitations of *Legionella* diagnostics have public health implications that include the inability to trace trends in disease incidence and delaying the recognition of outbreaks (12,242–245). Identifying an outbreak of *Legionella* is crucial, as typical outbreak strategies such as isolation of cases will not reduce cases, and the medium by which *Legionella* is contracted (water) makes outbreaks very likely and identification of common source exposure is essential to curtail the outbreak. The delay also contributes to increasing the mortality rate, as in immunosuppressed individuals the mortality rate can be up to 80% in infections across a range of different species of *Legionella* if left untreated (12,246). Furthermore, up to 90% of *Legionella* infections are missed even in environments that are equipped for diagnosis and the increasing mortality rate, warranting an increase in testing frequency (12). Our findings are in agreement with Decker et al., who found that systematically testing for *Legionella* in people diagnosed with pneumonia using two diagnostic techniques indicated that *Legionella* diagnoses had been underreported (241).

An opportunity for further research is to determine if PLHIV or other forms of immunosuppression have a higher susceptibility to specific subgroups of *Legionella*. While we had sought to investigate this, the lack of reporting of *Legionella* cases impeded our efforts in doing so. Head et al. found that 36% of PLHIV in their study population that was diagnosed with community-acquired pneumonia were coinfected with *Legionella* (246). In their study, approximately one-third of the *Legionella* infections were caused by *L. pneumophila*, none of which were LPSG1. However, it is unclear if these proportions are due to geographical factors or the presence of HIV. Sivagnanam et al. found that 31% and 47% of their American transplant recipient cohort were infected with *L. micdadei* and *L. pneumophila*, respectively (228). These studies emphasize the need to diversify diagnostic methods, especially in the immunocompromised population.

A limitation of our study is that further analyses cannot be performed because of a lack of information regarding the specific tests used, differences in populations tested, variable sample storage conditions, and that patients received inconsistent testing. Furthermore, studies used different techniques as their standards, only subjecting samples to further diagnosis with an initial positive result. Despite the heterogeneity in testing protocols and specific primers, PCR provided positive results in most tests. We were also unable to draw any conclusions about the effect of geographical distribution and regional

testing conventions with the number of studies included in our review. Additionally, researchers are more likely to publish results that reveal particularly high or low prevalence, which may cause intermediate cases to be unrepresented within the literature. This was also the reasoning behind our inclusion criteria "at least 5 cases of LD in the patient group", as articles reporting very few cases tended to be case reports/series or looking at isolated cases of LD without the generalized pneumonia context. Additionally, among the 30 studies that were excluded for the insufficient presence of *Legionella*, 15 found 0 cases of LD and 2 studies found 4 cases. The remaining 13 studies were also excluded for additional reasons, most frequently a lack of species/serogroup analysis and/or the absence of an NAT-based diagnosis.

In conclusion, the real epidemiology of *Legionella* infections is unclear due to the lack of an adequate diagnostic test that identifies other non-*pneumophila* serogroup 1 *Legionella* and different criteria on who, when, and how to diagnose *Legionella* (13,38,197,198,245). It is essential to isolate strains and carry out epidemiological research studies using whole genome sequencing to identify and track circulating strains of *Legionella* for future diagnostic test development, strains of concern, and clinical guideline updates.

Chapter 3: Growth curves and immunological patterns of clinical isolates of *Legionella* spp. in U937 cells

3.1 Introduction

Legionella is a family of gram-negative bacteria primarily found in water and soil. It has been known to cause respiratory infections in humans through the inhalation of contaminated water from both natural and man-made water systems, causing Legionnaires' disease (LD).

Individuals over the age of 45 are at a heightened risk of suffering a severe illness with a 10% mortality rate (11,247,248). A global aging population further exacerbates this. Another at-risk population for LD is people living with HIV (PLHIV) (249,250). Within this population, *Legionella* is capable of causing severe pneumonia that requires intensive care unit admission, with a mortality rate of 53% (249–252). Furthermore, total LD cases have drastically increased over time as a result of climate change. Warmer temperatures favour the growth of *Legionella*'s environmental amoebal hosts resulting in increased tropical storm intensity causing contamination of water reservoirs, positing *Legionella* as the leading cause of non-enteric infections in post-storm settings (32).

Historically, *Legionella pneumophila* serogroup 1 has been thought to cause approximately 80% of *Legionella* infections, but evidence has shown that nearly 50% of LD cases are caused by unspecified *Legionella* species or serogroups not detected by the standard urinary antigen test, and regional differences in etiology have been observed such as the high presence of *L*. *longbeachae* in New Zealand and Australia at 63% and 59%, respectively (2,17,70,251). The widely used BinaxNOW urinary antigen test is specific for identifying *L. pneumophila* serogroup 1 has limited detection rates for other species and serogroups of *Legionella*. Culture, the gold standard of Legionella diagnostics, has proven difficult in clinical samples with less than half of LD patients producing sputum and less than 44% of LD patients are diagnosed by sputum culture (253–256) . The difficulties in identifying and recovering *Legionella* isolates from patients limit the strains available to study in vitro such as infection assays and transcriptomics that can improve our understanding of *Legionella's* pathogenesis.

Legionella's primary target in human hosts is phagocytic cells such as macrophages and monocytes, which play a critical role in initiating the immune response. Alveolar macrophages serve as a key antigen-presenting cell in the early stages of the innate immune response, bridging the innate immune response to the adaptive. When faced with intracellular pathogens such as *Legionella*, phagocytes are hijacked for use as reservoirs, facilitating pathogen replication (2,17). *Legionella*'s ability to survive in intracellular

space and circumvent intracellular lysis creates a scenario in which cell-mediated killing is the predominant method of controlling the infection (97,257). Thus, cytokine responses exhibited by these key mediators in the immune system are important in understanding the pathogenesis of intra-cellular pathogens such as *Legionella*.

For this reason, we investigated the intracellular growth of *L. bozemanae*, *L. dumoffii*, *L. micdadei*, and *L. pneumophila* in a U937 cell model. Then we studied the cytokine profiles elicited by U937 cells after infection with *Legionella* sp. The examined cytokines play roles in inflammation, chemotaxis, and anti-inflammation. Given the diversity in antigen expression, effector proteins, and physiology found in *Legionella* species, we hypothesized that each species would elicit distinct immune responses (141,258,259).

3.2 Methods

3.2.1 *Legionella* Cultures

L. pneumophila Philadelphia-1 (ATCC), *L. bozemanae* (clinical strain from Winnipeg, Manitoba), *L. dumoffii* (clinical strain from Winnipeg, Manitoba), *L. micdadei* (clinical strain from Winnipeg, Manitoba), and *L. pneumophila* (clinical strain from Winnipeg, Manitoba) were grown in buffered yeast charcoal extract (BCYE) agar for 5 days at 37˚C in 5% CO_2. Colonies were then picked and grown in buffered yeast extract broth for 18 hours at 37˚C to allow cells to reach the stationary phase. The detailed protocol of the culture and infection is reported in supplementary material 1.

3.2.2 Maintaining U937 Cells

Monocytic U937 cells purchased from the American Type Culture Collection (ATCC, ATCC code: CRL-1593.2) were maintained at 37˚C in 5% CO_2 using Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% Fetal bovine serum (FBS, Wisent, Canada) and 2% Penicillin-Streptomycin (Sigma Aldrich, USA). Cells were passaged 3-5 times from thaw before phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, United States) treatment. Cell viability and counts were determined using a hemocytometer and trypan blue. Cells were seeded in a 24-well plate at a density of $1x10^6$ cells/mL and treated with 50ng/mL PMA for 24 hours before washing.

3.2.3 U937 Infection

Using the ratio 1OD600: 1x109 cells/mL, U937 cells were infected at a multiplicity of infection of 2 in RPMI1640 with 10% FBS. The cell-bacteria mixture was incubated for 1hr at 37˚C in 5% CO2 followed by two washes with RPMI1640 with 10% FBS to remove non-internalized bacteria. The washed cells were then incubated for 1hr at 37˚C in 5% CO2 with 100µg/mL gentamicin for 0h, 4, 24h, and 48h. Post-gentamicin incubation, each well was washed thrice with RPMI and then incubated at 37˚C in 5% CO2 until the respective lysis timepoint. Uninfected negative controls underwent the same treatments using RPMI1640 with 10% FBS and no bacteria. Infections were done in triplicate.

3.2.4 Cellular Lysis

At 0h, 4h, 24h, and 48h timepoints, the supernatant was removed from each well, stored at -20˚C for later cytokine analysis, and then replaced with 0.1% Tween20-PBS (Sigma, United States; Wisent, Canada). The bottoms of each well were scraped with a pipet tip and then incubated at room temperature for 25 minutes before being scraped again.

3.2.5 Bacterial Growth Curves

Cell lysates were diluted in a 10-fold series, then plated on BCYE agar for 4-5 days before colonies were counted. Colony counts were used to construct a growth curve. Where colonies were countable on multiple dilutions, the least diluted plates were counted.

3.2.6 Cytokine Selection

We used several strategies to select the cytokines to be studied: a) a literature review was conducted to identify cytokines studied in *Legionella* infections (*in vitro*, animal models and humans) in cell culture, whole blood, or plasma; b) cytokines produced by macrophages and monocytes upon infection by other intracellular pathogens, c) based on the availability of antibodies for detecting said cytokines in commercial kits (Table 8). Selected cytokines were then categorized into three categories based on their primary role during inflammation: activation, chemotaxis, and anti-inflammation. Anti-inflammatory cytokines were included due to *Legionella*'s host modulation capabilities and eukaryote lipid hijacking as methods to dampen immune responses (136,260,261). The final panel of cytokines included Eotaxin, FGF-2 (fibroblast growth factor-2), Fractalkine, GM-CSF (Granulocyte/macrophage colony-stimulating factor), IFN (interferon)-α2, IFN-γ, IL (interleukin)-1β, IL-6, IL-8, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IP (interferon gamma-induced protein)-10, MCP (monocyte chemoattractant protein)-1, MCP-2, RANTES (regulated upon activation, normal T-cell expressed and secreted), TGF (transforming growth factor)-β, and TNF (tumour necrosis factor)-α.

Table 8. Cytokines hypothesized to be relevant in *Legionella* infections and their immunological roles, immune-relevant pathways markers are involved in and studies, part 1 of 6.

Marker (and common alternative names)	Activation	Chemotaxis	Anti-inflammation	Studied in Legionella infections?	Observed in macrophages /monocytes	Studied in animal cells/models	Studied in vitro?	Studied from clinical samples?	(KEGG: Kyoto Encyclopedia of Genes and Genomes) entry code(s)	Relevant Pathways/Processes
Eotaxin (CCL11, CCL24, CCL26)	(262,263)			(250)			(264)	(250)	hsa:6356, hsa:6369, hsa:10344	Cytokine-cytokine receptor, viral protein interaction with cytokine and cytokine receptor, chemokine signaling, IL-17 signaling
FGF-2	(265,266)				(265)		(265)		hsa:2247	MAPK, Ras, Rap1, Ca^{2+} signaling, PI3K-akt, ERK signaling
Fractalkine (CX3CL1, CXC3C)		(267)						(268)	hsa:6376	signal transduction (GPCR, rho), cytokine-cytokine receptor interaction, viral protein interaction with cytokine and receptor, chemokine signaling, TNF signaling, efferocytosis, CXCR1-GNAI-AC-PKA signaling
GM-CSF (CSF2, CSF)	(269)			(270–272)	(272)	(270,272)	(270)		hsa:1437	PI3K-αkt, Raf-Ras, JAK/STAT, cytokine-cytokine receptor interaction, IL-17 signaling, TCR signaling, FcERI signaling, TNF signaling, NK-mediated cytotoxicity
IFN-α2	(273)			(175,274,275)	(175,274)	(175,275)		(268)	hsa:3440	PI3K, cytokine-cytokine receptor interaction, necroptosis, TLR, NOD-LR, RIG, cytosolic DNA sensing, JAK/STAT, NK-mediated cytotoxicity

Continuation of table 8, part 2 of 6

Marker (and common alternative names)	Activation	Chemotaxis	Anti-inflammation	Studied in Legionella infections?	Observed in macrophages /monocytes	Studied in animal cells/models	Studied in vitro?	Studied from clinical samples?	(KEGG: Kyoto Encyclopedia of Genes and Genomes) entry code(s)	Relevant Pathways/Processes
IFN-γ	(276)			(175,275,277–279)	(175)	(175,275,277,278)	(277)	(268,279)	hsa:3458	Proteasome, cytokine-cytokine receptor interaction, HIF-1 signaling pathway, necroptosis, TGF-beta signaling pathway, antigen processing and presentation, JAK-STAT signaling, NK cell mediated cytotoxicity, IL-17 signaling, Th1 and Th2 cell differentiation, Th17 cell differentiation, TCR signaling
IL-1β	(280)			(88,270)	(270,281,282)	(270)(270)	(88,270)	(268)	hsa:3553	MAPK signaling, cytokine-cytokine receptor, NFkB signaling, necroptosis, TLR signaling, NOD-like receptor signaling, cytosolic DNA-sensing signaling, IL-17 signaling, hematopoietic cell lineage, Th17 cell differentiation, TNF signaling, inflammatory mediator of TRP channels
IL-6 (BSF2, IFN-b2)		(283)		(88,270,284)	(88,270,285)	(270)	(88,270,284)	(268)	hsa:3569	HIF-1, FoxO, PI3K, TLR, NOD, JAK-STAT, IL-17, cellular senescence, cytosolic DNA-sensing, C-type lectin receptor signaling, Th17 cell differentiation

Continuation of table 8, part 3 of 6

Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)
IL-8 (CXCL8, GCP-1)		(286)		(88,270)	(88)		(88,270)	(268)	hsa:3576	Cytokine-cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptor, chemokine signaling, NFkB signaling, phospholipase D signaling, cellular senescence, TLR signaling, NOD-like signaling, RIG-I-like receptor signaling, IL-17 signaling, Legionellosis
IL-9 (P40)	(287)				(288)	(289)		(288,290)	hsa:3578	Cytokine-cytokine receptor, JAK/STAT
IL-10 (CSIF, TGIF)			(100,278)	(88,100,277)	(88,277,278)	(278)	(88,277, 278)	(291)	hsa:3586	Cytokine-cytokine receptor, viral protein interaction with cytokine and cytokine receptor, FoxO signaling, efferocytosis, C-type lectin receptor signaling, JAK-STAT signaling
IL-12 (IL-12p40 & IL-12p70, collectively)	(292)			(278)	(269)	(278)	(293)	(279)	hsa:3592, hsa:3593	Cytokine-cytokine receptor, TLR signaling, RIG-I-like receptor signaling, C-type lectin receptor signaling, JAK-STAT signaling, Th1/Th2 differentiation, Legionellosis
IL-13 (P600)			(294)		(295)	(295)			hsa:3596	cytokine-cytokine receptor interaction, JAK-STAT signaling, IL-17 signaling, Th1/2 differentiation, FcεRI signaling

Continuation of table 8, part 4 of 6

Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)
IP-10 (CXCL10, SCYB10, INP10)		(101,102)		(250)				(250,296)	hsa:3627	Cytokine-cytokine receptor interaction, viral protein interaction with cytokine-cytokine receptor, chemokine signaling, TLR signaling, RIG-I-like receptor signaling, Cytosolic DNA-sensing, IL-17 signaling, TNF signaling
MCP-1 (CCL2, GDCF-2, SCYA2)		(100)		(88,100,250, 278)	(88,100)	(100,278)	(88,100)	(250)	hsa:6347	Cytokine-cytokine receptor interaction, viral protein interaction with cytokine-cytokine receptor, chemokine signaling, NOD-like receptor signaling, IL-17 signaling, TNF signaling
MCP-2 (CCL8, SCYA10, SCYA8)		(99)		(88,278)		(278)	(88)		hsa:6355	Cytokine-cytokine receptor interaction, viral protein recognition, chemokine signaling
RANTES (CCL5, SCYA5)		(297)				(278,298)		(296)	hsa:6352	Cytokine-cytokine receptor interaction, viral protein interaction with cytokine-cytokine receptor, chemokine signaling, TLR signaling, NOD-like receptor signaling, cytosolic DNA-sensing, TNF signaling

Continuation of table 8, part 5 of 6

Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)
TGF-β			(299)	(300)	(300)		(300)		hsa:7040, hsa:7042, hsa:7043	MAPK signaling, cytokine-cytokine receptor interaction, FoxO signaling, cell cycle, cellular senescence, TGF-β signaling, osteoclast differentiation, Hippo signaling, cancer (proteoglycans, microRNA), efferocytosis, Th17 cell differentiation, relaxin signaling

Continuation of table 8, part 6 of 6

Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)	Marker (and common alternative names)
TNF-α	(301)			(88,279,300)	(88,285,300)	(302)	(88,285, 300)	(279)	hsa:7124	MAPK signaling, cytokine-cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptor, NFkB signaling, sphingolipid signaling, mTOR signaling, apoptosis, necroptosis, TGF-beta signaling, antigen processing and presentation, TLR signaling, NOD-like receptor signaling, RIG-I-like receptor signaling, C-type lectin receptor signaling, hematopoietic cell lineage, NK cell-mediated cytotoxicity, IL-17 signaling, TCR signaling, FceRI signaling, TNF signaling, adipocytokine signaling, Legionellosis

Reactome.org does not differentiate IL-12p40 and IL-12p70. *simian IL-8 pathways. **kegg.jp separates TGF-β into entries, one for each isomer. ***TGF-β1-exclusive. Kegg.jp entries for TGF-β2/3 list the same pathways. List of pathways was compiled based on information found on kegg.jp and reactome.org.

3.2.7 Cytokine Quantity Evaluation

Frozen tissue culture supernatant was thawed, and cytokine concentrations were measured using MILLIPLEX® Human Cytokine/Chemokine Magnetic Bead Panels A, B, and TGFβ1 Single Plex (Sigma-Aldrich, United States) according to manufacturer instructions using a Luminex™ 200™ (Luminex, Belgium) platform and the xPONENT® Software Solutions (Luminex, Belgium). We followed protocols using overnight incubation and substituted sheath fluid for PBS at the sample reading step. Any readings below the limit of detection (LOD) were assigned a value equal to 0.5*(LOD) for the respective analyte. LOD values were as follows: Eotaxin, 3pg/mL; FGF-2, 26pg/mL; fractalkine, 32pg/mL; GM-CSF, 2.6pg/mL; IFN-α2, 8pg/mL; IFN-γ, 1.3pg/mL; IL-1β, 1.6pg/mL; IL-6, 0.64pg/mL; IL-8, 0.64pg/mL; IL-9, 0.64pg/mL; IL-10, 2.6pg/mL; IL-12p40, 6.4pg/mL; IL-12p70, 3pg/mL; IL-13, 6.4pg/mL; IP-10, 2.6pg/mL; MCP-1, 3pg/mL; MCP-2, 0.2pg/mL; RANTES, 1.3pg/mL; TGF-β1, 9.8pg/mL; TNF-α, 6.4pg/mL.

3.2.8 Statistical Analysis

Fold changes were calculated relative to the 0-hour (baseline) expression of each cytokine within the same infection condition. These figures were used to summarize the results of all cytokines studied in each infection condition. IFN-α2, fractalkine, IL-12p40, and IL-12p70 were all expressed at levels below the LOD, and therefore, these immune markers were not included in the analysis. t-tests were conducted using standard error of means to determine the significance of a cytokine measure of infected cell conditions relative to the time-equivalent uninfected condition.

3.3 Results

The intracellular growth rate varied among species and strains of *Legionella* (Fig 2). The fastest growth rate was observed in the clinical *L. pneumophila* (Fig 2A) strain followed by *L. dumoffii* (Fig 1C), *L. pneumophila* Philadelphia-1 (Fig 2E), *L. micdadei* (Fig 2D), and *L. bozemanae* (Fig 2B) was observed to have very slow intracellular growth over the 48h infection period. *L. micdadei* showed minimal internalization and initial growth at the 0h and 4h timepoints, respectively, yielding no colonies. However, the recovered CFU at the 24h and 48h timepoints was comparable to that of *L. pneumophila* Philadelphia-1 (Fig 2D, 2E). The other strains showed robust growth, with the clinical strains of *L. dumoffii* and *L. pneumophila* outgrowing the ATCC strain, *L. pneumophila* Philadelphia-1 by approximately 1 to 1.5 orders of magnitude (Fig 2A, 2B, 2E).

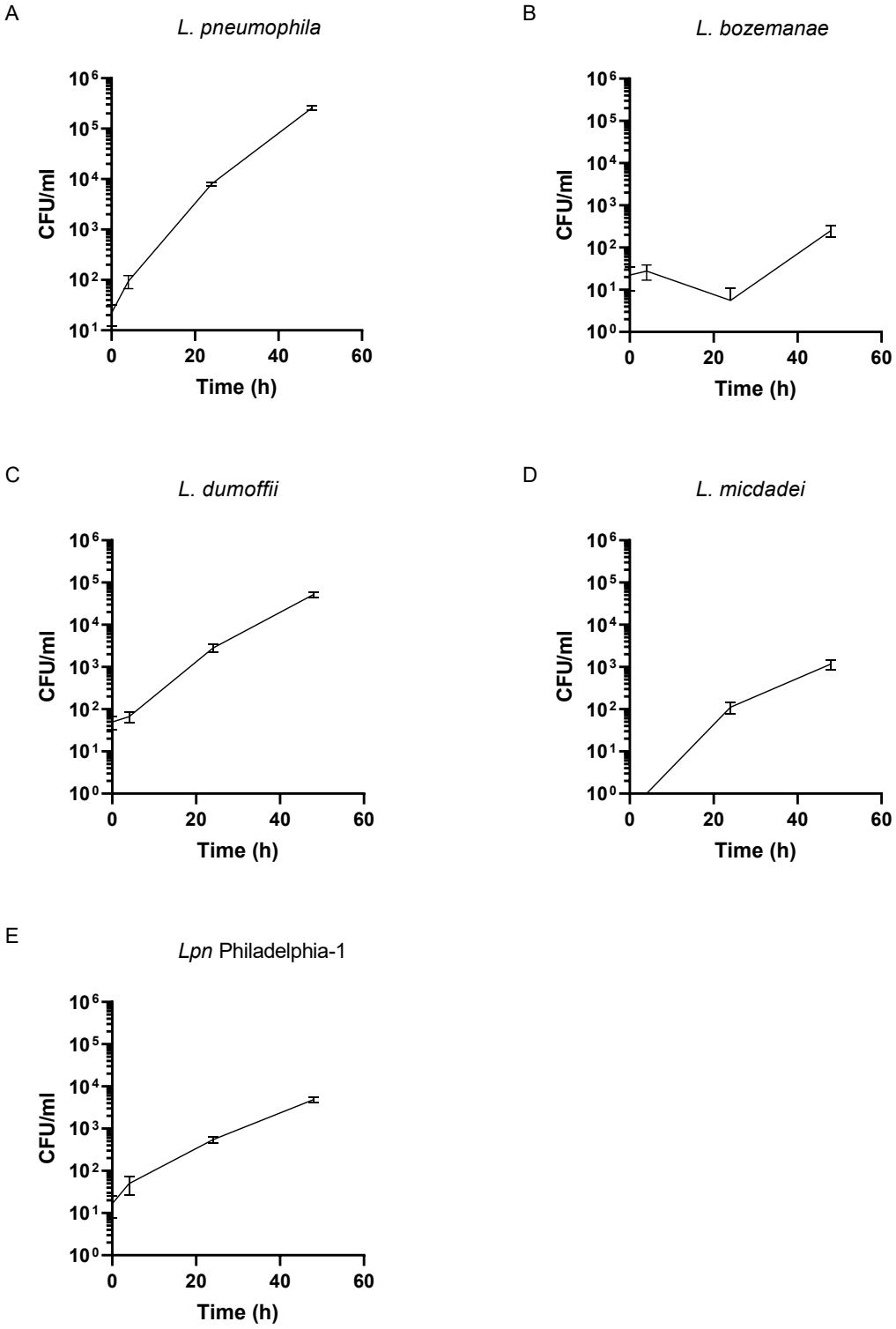
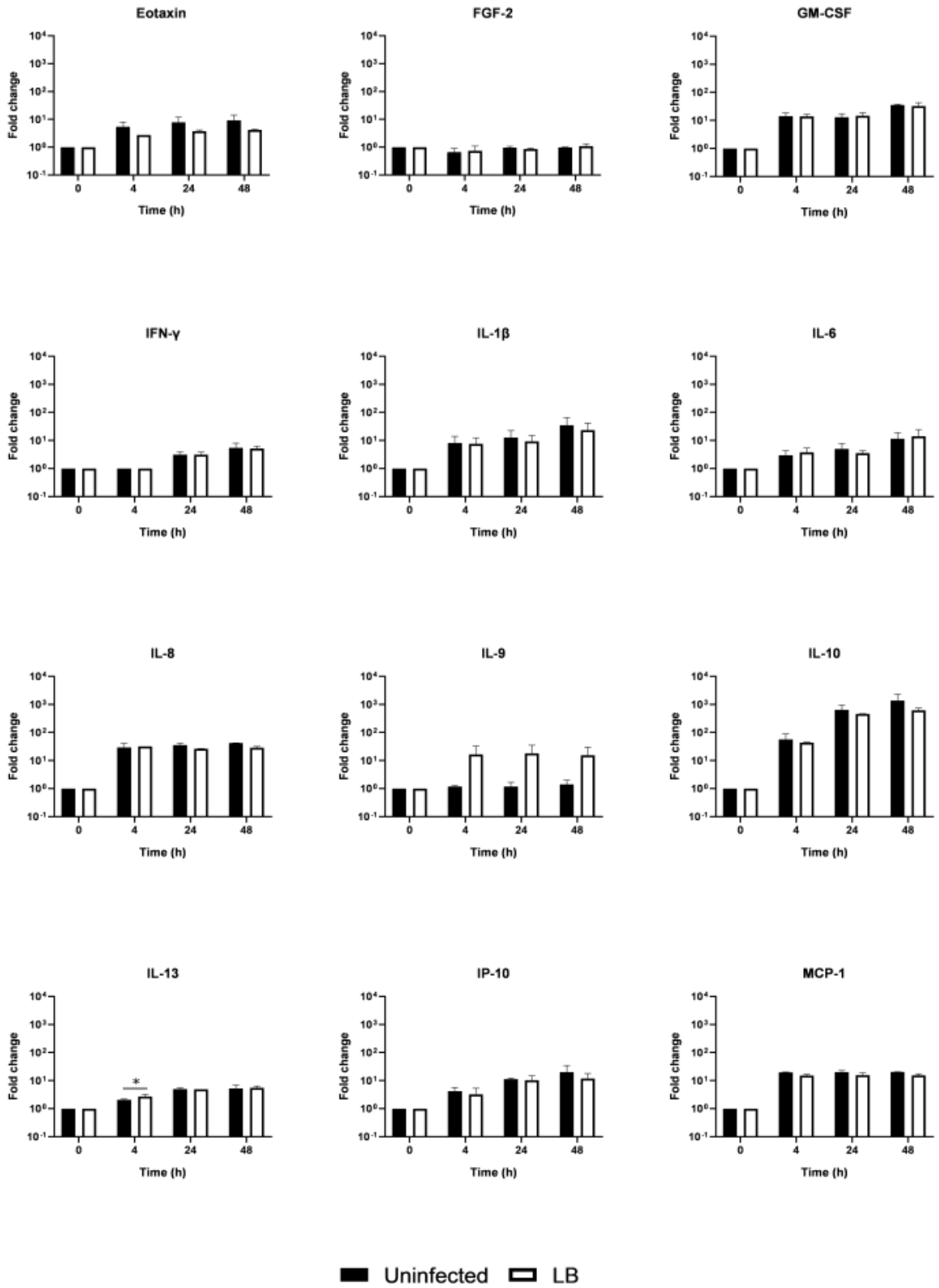


Figure 2. *Legionella* recovered from infection of U937 cells on BCYE agar. Experiments were done in triplicate.

Upon analyzing fold changes in cytokine expression relative to 0-hour baseline expression, proinflammatory markers largely saw downregulation across infections with all species of *Legionella* while anti-inflammatory markers increased in expression. No significant changes were seen in expression at 24 hours post-infection. Few changes in expression were seen at 4 hours post-infection, namely, IL-13 and TGF-β which increased in *L. bozemanae*-infected (p=0.0223, p=0.0278); and FGF-2 and IL-13 which increased in *L. pneumophila* Philadelphia-1-infected cells (p=0.0208, p=0.0113) (Figs 3,4). *L. pneumophila*-infected cells expressed changes in the expression of 3 different cytokines relative to uninfected controls, a broader change in response than other examined conditions, all at 48 hours post-infection. These are FGF-2, GM-CSF, and IL-8 (p=0.0027, p=0.0277, p=0.0069), all decreasing relative to the uninfected cell expression (Fig 5). Coincidentally, this infection condition yielded the highest CFU/mL (Fig 2). At 48 hours *L. pneumophila* Philadelphia-1-infected cells increased expression of FGF-2 (p=0.0004) (Fig 4). *L. dumoffii*-infected cells expressed decreased GM-CSF at 48 hours post-infection (p=0.0277) (Fig 6). *L. bozemanae*-infected cells increased expression of TGF-β at 48 hours post-infection (p=0.0278) (Fig 3). And *L micdadei*-infected cells increased expression of MCP-2 48 hours post-infection (p=0.021) (Fig 7). All changes are relative to uninfected controls.


Eotaxin
FGF-2
GM-CSF
IFN-γ
IL-1β
IL-6
IL-8
IL-9
IL-10
IL-13
IP-10
MCP-1
Fold change
Time (h)
0
4
24
48
*
Uninfected
LB

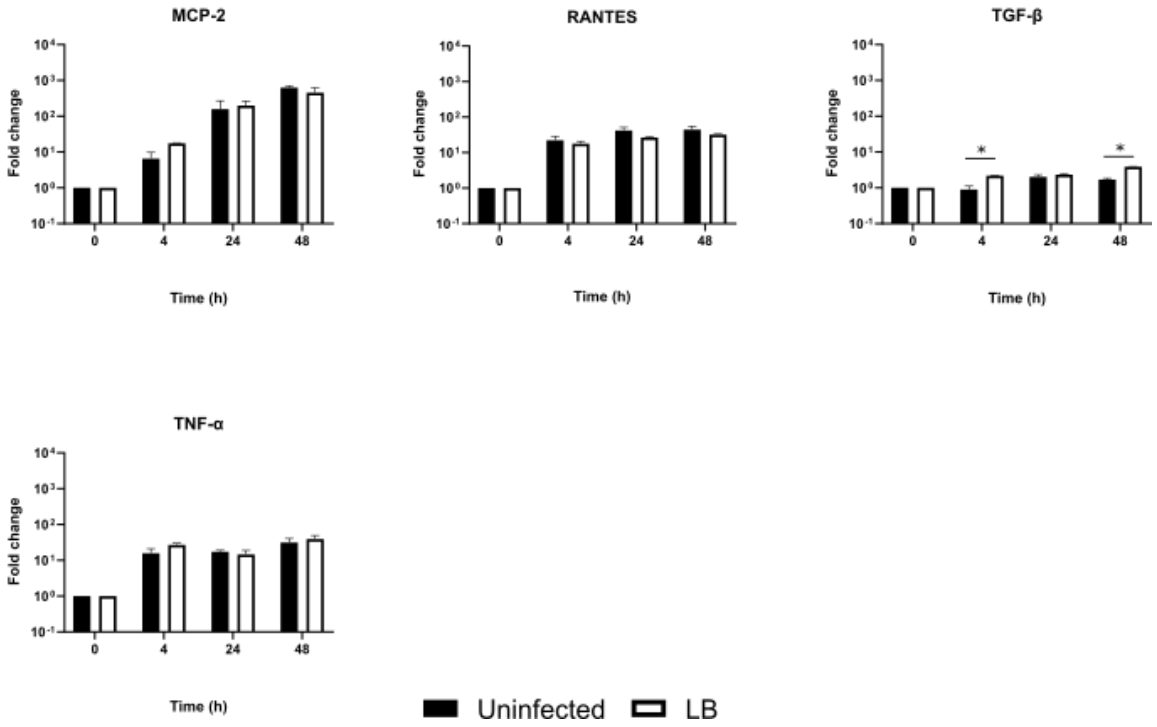
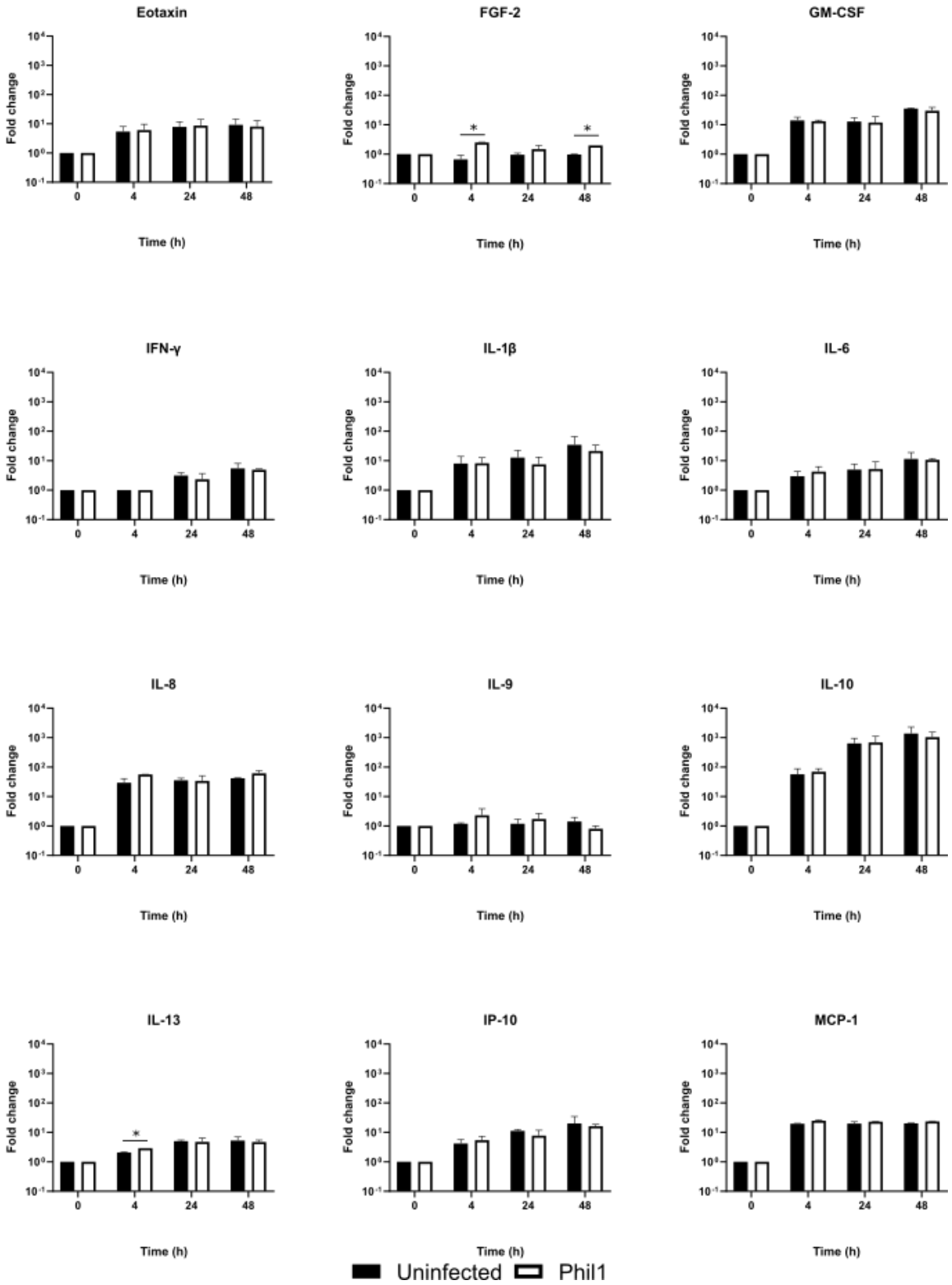


Figure 3. Fold change cytokine expression in *L. bozemanae*-infected U937 relative to 0-hour expression under the same culturing conditions. LB, *L. bozemanae*. *, $p < 0.05$ compared with negative control using t-test.


Eotaxin
FGF-2
GM-CSF
IFN-γ
IL-1β
IL-6
IL-8
IL-9
IL-10
IL-13
IP-10
MCP-1
Fold change
Time (h)
0
4
24
48
*
Uninfected
Phil1

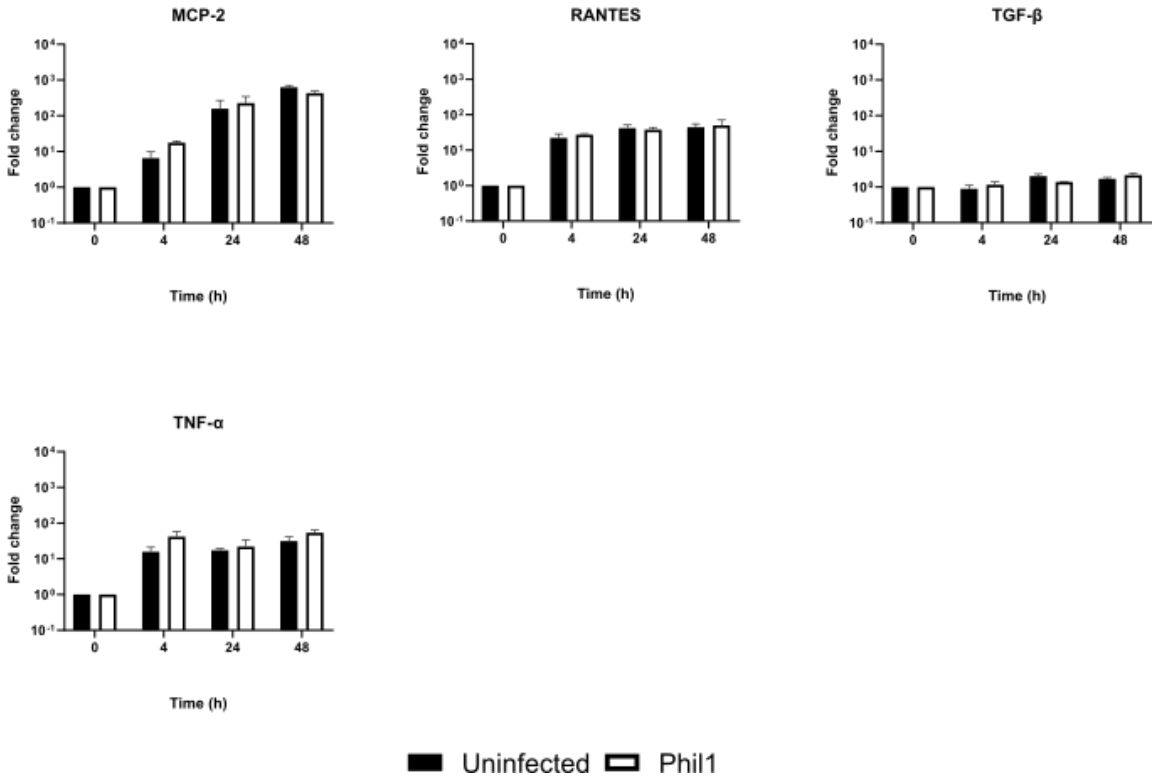
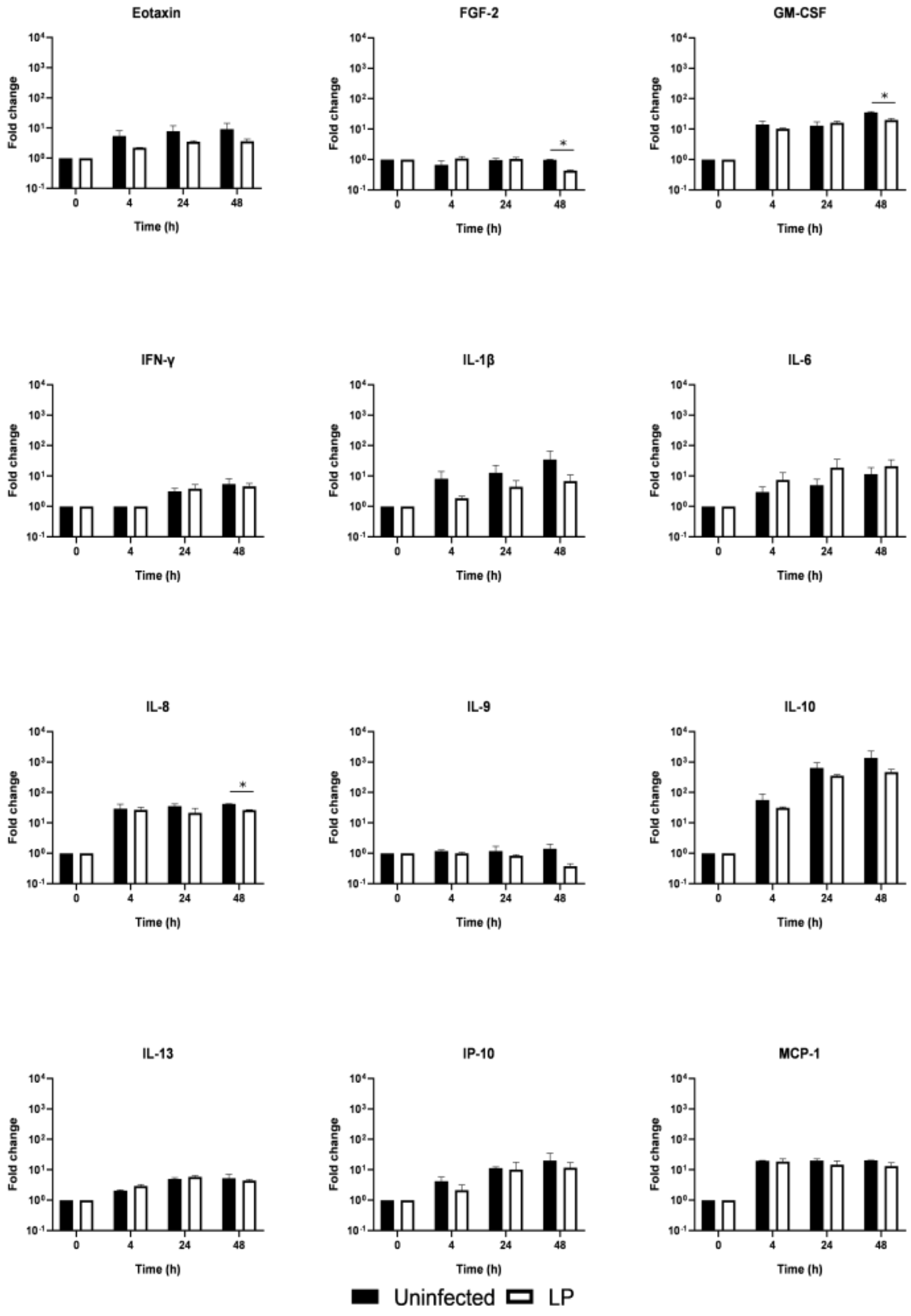


Figure 4. Fold change cytokine expression in *L. pneumophila* Philadelphia-1-infected U937 relative to 0-hour expression under the same culturing conditions. Phil1, *L. pneumophila* Philadelphia-1. **,* $p < 0.05$ compared with negative control using t-test.


Eotaxin
FGF-2
GM-CSF
IFN-γ
IL-1β
IL-6
IL-8
IL-9
IL-10
IL-13
IP-10
MCP-1
Fold change
Time (h)
0
4
24
48
*
Uninfected
LP

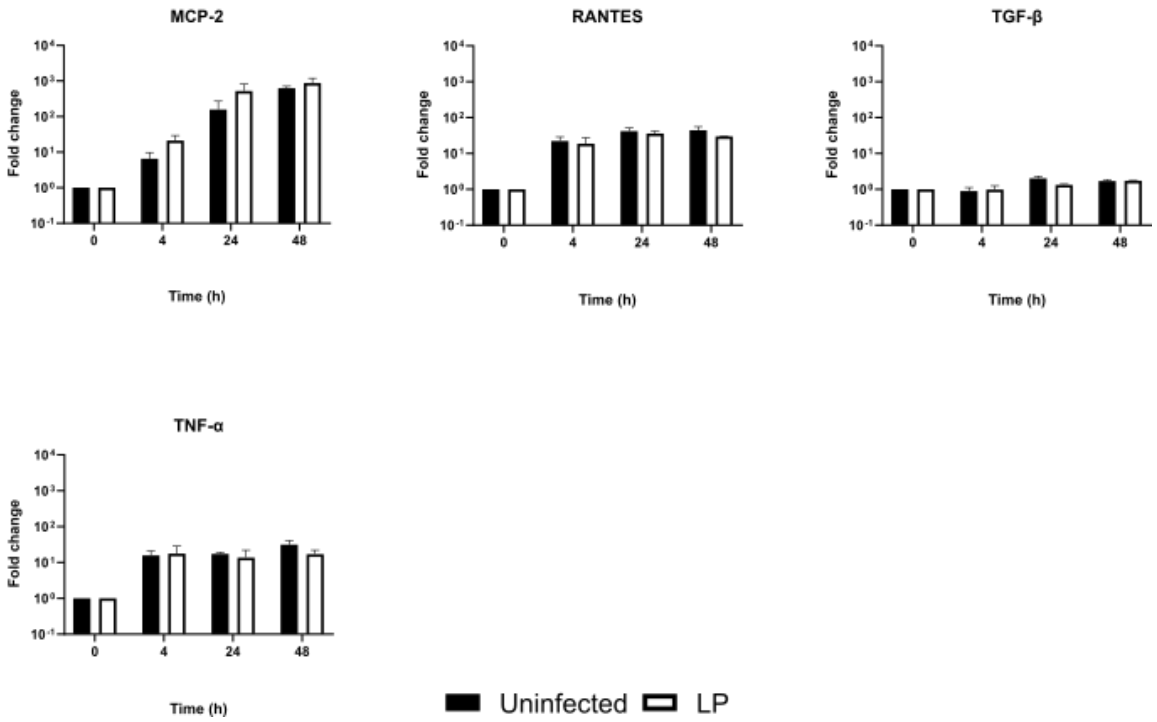
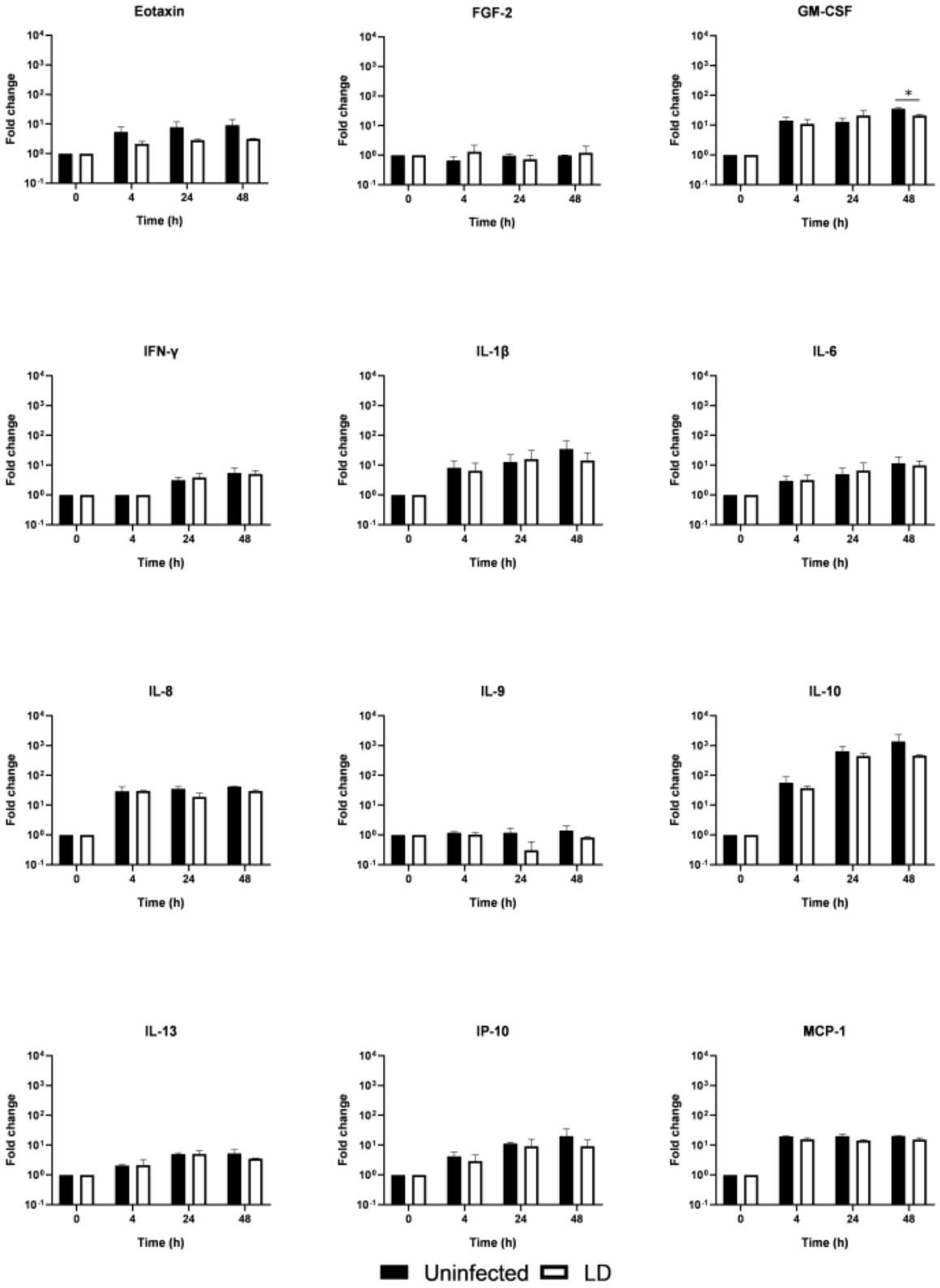


Figure 5. Fold change cytokine expression in *L. pneumophila*-infected U937 relative to 0-hour expression under the same culturing conditions. LP, *L. pneumophila*. *, $p < 0.05$ compared with negative control using t-test.


Eotaxin
FGF-2
GM-CSF
IFN-γ
IL-1β
IL-6
IL-8
IL-9
IL-10
IL-13
IP-10
MCP-1
Fold change
Time (h)
0
4
24
48
*
Uninfected
LD

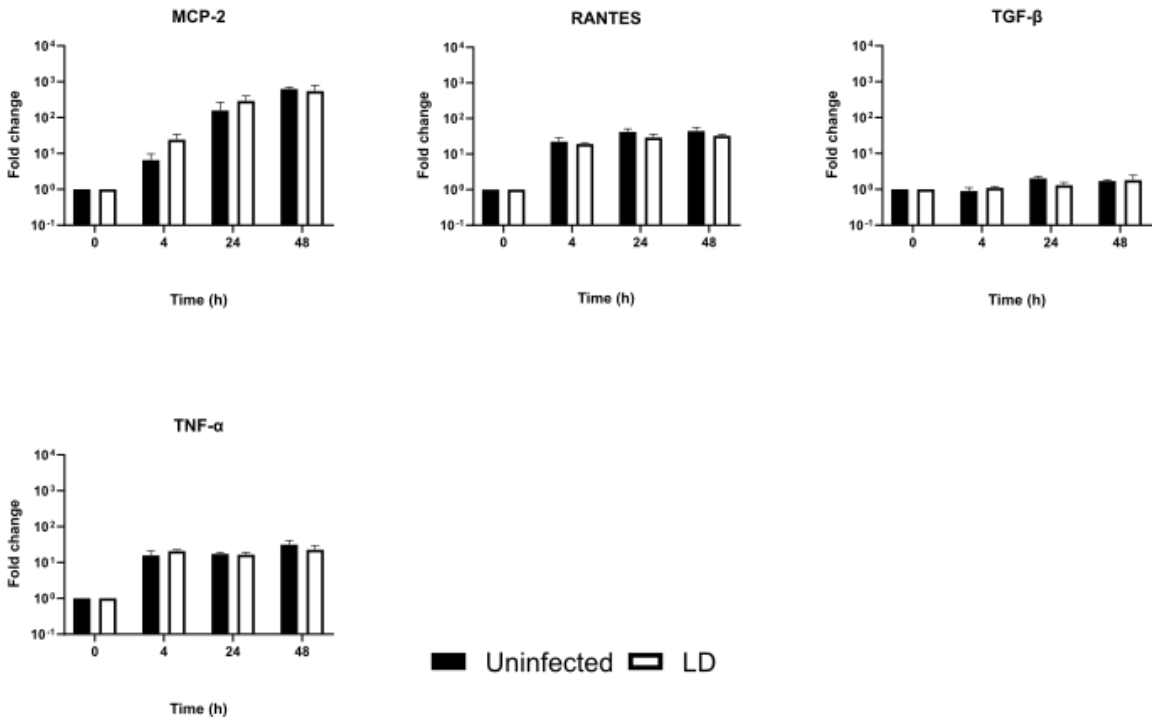
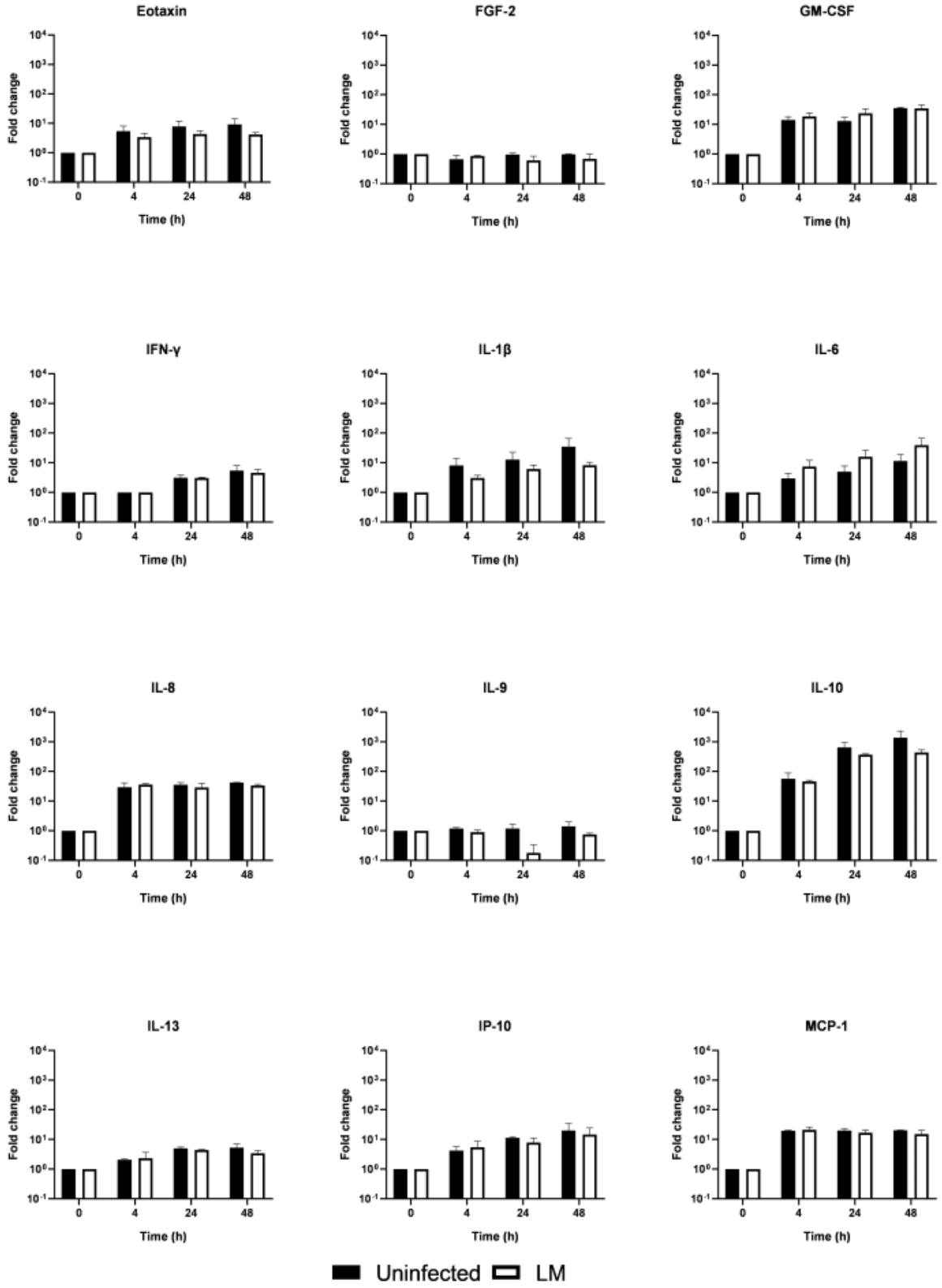


Figure 6. Fold change cytokine expression in *L. dumoffii*-infected U937 relative to 0-hour expression under the same culturing conditions. LD, *L. dumoffii*. *, $p < 0.05$ compared with negative control using t-test.


Eotaxin
FGF-2
GM-CSF
IFN-γ
IL-1β
IL-6
IL-8
IL-9
IL-10
IL-13
IP-10
MCP-1
Fold change
Time (h)
0
4
24
48
Uninfected
LM

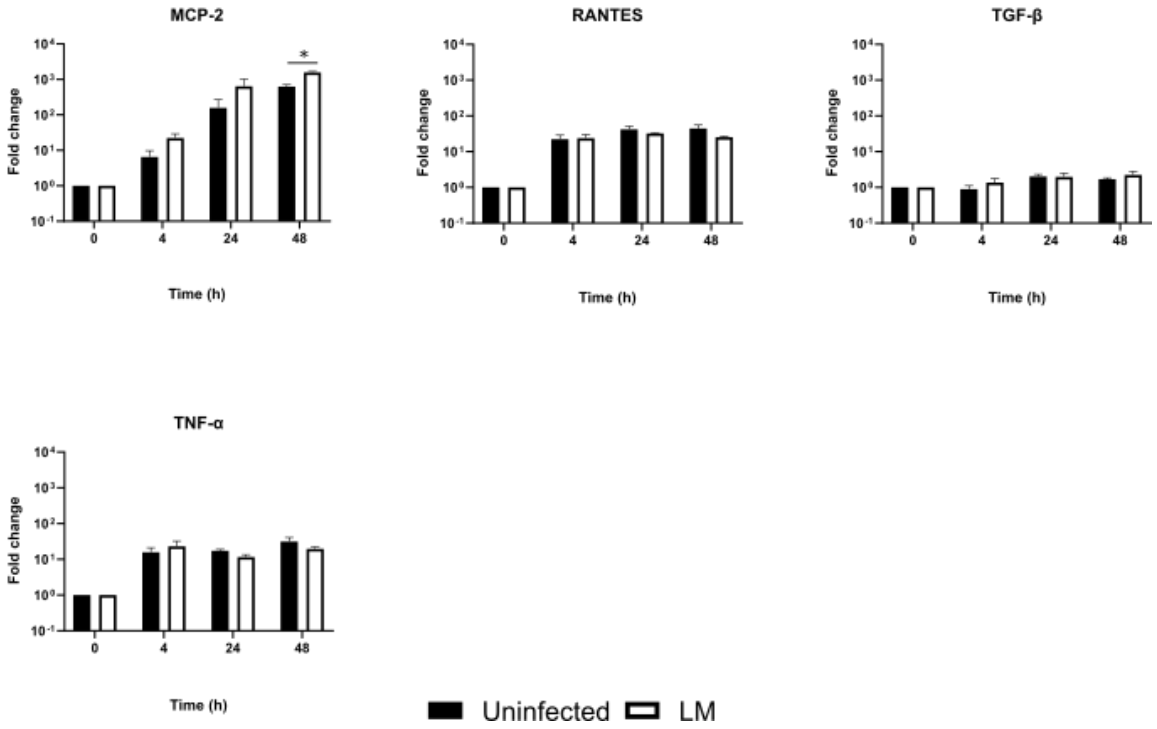


Figure 7. Fold change cytokine expression in *L. micdadei*-infected U937 relative to 0-hour expression under the same culturing conditions. LM, *L. micdadei*. *, $p < 0.05$ compared with negative control using t-test.

3.4 Discussion

With the increasing burden of *Legionella* infections and modern studies showing an increased representation of non-*pneumophila Legionella* species in *Legionella* pneumonia, it is important to understand the mechanisms of pathogenesis in these understudied species (10,11,17,251). Here, we show the cytokine response mounted by a human monocytic cell line upon infection with clinical isolates of *Legionella* obtained from a tertiary care hospital in Winnipeg, Canada.

It is unclear if differences in intracellular growth rate result from differences in infectivity or degree of host modification. Park *et al.* showed several other less common species of *Legionella* with growth rates comparable to *L. pneumophila* such as *L. tucsonensis*, *L. spiritensis*, *L. micdadei*, and *L. hackeliae* (118). Park et al.'s findings after 36h of infection are similar to our own, showing that *L. dumoffii* grew faster than *L. pneumophila* Philadelphia-1 and *L. micdadei*'s growth rate being similar to *L. pneumophila* Philadelphia-1 at both 24h and 48h timepoints (118). *L. dumoffii* has been reported to have a faster intracellular replication rate compared to *L. pneumophila* in U937 cells (118). However, this has been disputed by another report that the replication rates are similar in Mono Mac 6 cells, but *L. dumoffii* has a higher rate of initial cellular entry (118,300,303). *L. micdadei*'s slow early growth, but sudden quick growth to 10^2CFU/mL and 10^3CFU/mL observed at the 24h and 48h timepoints suggests that physiological changes occur before 24h to prepare for intracellular growth. It is plausible that this may be a result of delayed release of host-modulating effector protein by *L. micdadei* compared to species with earlier growth (304). It is conceivable that effector proteins disrupting phagolysosome fusion are released early to facilitate survival, but nutrient trafficking effectors have a delayed release (134,145,152,154). Despite the high infectivity and replicability in human cells relative to most other species suggesting that *L. dumoffii* and *L. micdadei* are sufficiently capable of replicating within human alveolar macrophages, reaching a final CFU/mL over 10^3, these species are infrequently identified in clinical contexts (2). In future studies under these conditions, *L. bozemanae* can be grown for longer to allow for extended measurements, possibly intensifying effects on the immune response and earlier timepoints can be incorporated between 4h and 24h to better understand the lag in *L. micdadei*'s intracellular growth.

The cytokines affected by *Legionella* infection in this study have been observed in other studies studying immune responses to *Legionella* spp. (88,270,300,305). Figure 8 depicts a schematic summary of *Legionella* pathogenesis based on our findings and previously published literature.

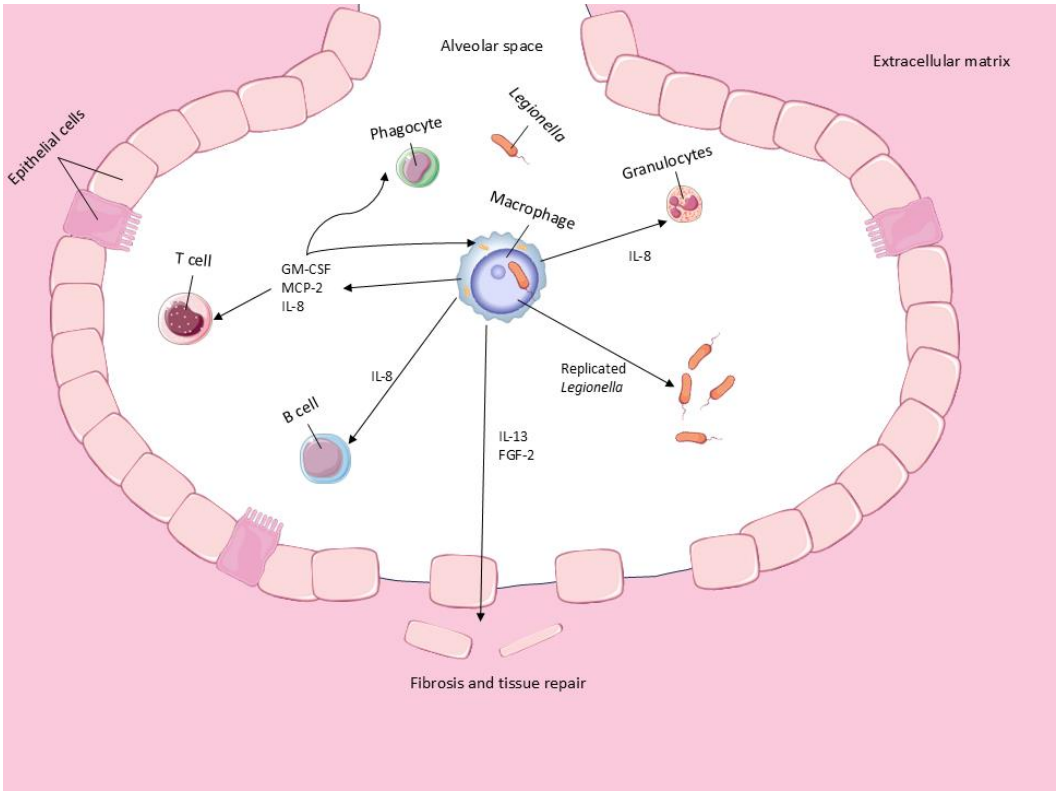


Figure 8. Proposed framework of *Legionella* pathogenesis based on the findings within this paper. Composed of images obtained from Servier Medical Art and SciDraw.

The magnitude of measured cytokine response to infections does not appear to be correlated with bacterial count (Fig 2). One potential explanation for this observation is that our working model may have residual antibiotics within cells from culturing steps before infection that may impact the course of infection by restricting bacterial numbers, and consequently immune response.

L. micdadei-, *L. bozemanae*-, and *L. pneumophila* Philadelphia-1-infected cells only experienced increases in cytokine expression. These strains also experienced the least intracellular growth within U937 cells (Fig 2). In contrast, *L. pneumophila* and *L. dumoffii* experienced the highest intracellular growth of around 10^5CFU/mL and exclusively caused decreases in cytokine expression.

Anti-inflammatory cytokines, namely TGF-β and IL-13, were significantly increased in *L. bozemanae*-infected cells, in which the expression increased relative to uninfected controls. This may result from *L. bozemanae*'s low replication capacity in U937 cells (Fig 8). Other infection conditions resulted in no significant increases or decreases in anti-inflammatory cytokines. It may be hypothesized that *L. bozemanae*'s low replication causes host cells to express an anti-inflammatory response. Proinflammatory markers FGF-2, GM-CSF, and IL-8 were downregulated in *L. pneumophila*- and *L. dumoffii*-infected cells, the strains that expressed the most intracellular growth. These cytokines play roles in immune activation and chemotaxis (Table 8). Disruption of these key early immune response events is crucial to the survival and proliferation of *Legionella* (120,306).

Despite both achieving a low final CFU/ml of approximately 10^3, *L. bozemanae* and *L. micdadei* induced opposite responses, anti-inflammatory and proinflammatory, respectively. This may be due to the differences in the early intracellular replication. *L. bozemanae*'s intracellular CFU/mL remained stagnant prior to the 48-hour reading (Fig 2). This contrasts the patterns seen in *L. micdadei*'s intracellular CFU/mL, which consistently increased over the same period (Fig 2). Thus, it may be hypothesized that *L. bozemanae*'s induction of TGF-β and IL-13 expression and the *L. micdadei*-induced increase in MCP-2 are caused by the early patterns in *Legionella* growth.

Among the strains of *Legionella* used in this study, *L. pneumphila* Philadelphia-1 is the only non-clinical strain and uniquely caused increases in inflammatory cytokine expression despite robust intracellular growth. This can be hypothesized as being caused by its status as a type strain, having been propagated for generations without conditions selecting for all of its virulence factors.

L. dumoffii- and *L. pneumophila*-infected cells had reduced secretion of GM-CSF, in contrast to the observations by Yamamoto et al. using mouse macrophages (270). It is unclear what reduced GM-CSF

contributes to either the pathology of *Legionella* infection or host response, as it has been reported to be a bystander in mouse macrophages (307). However, existing evidence hints at GM-CSF having more pronounced effects in more complex models, in which it increases cytokine production in response to pathogen recognition through JAK-STAT (Janus kinase-signal transducer and activators of transcription) pathways as noted by Liu et al. (272). Reduced GM-CSF can be assumed to play a paradoxical role, impeding the clearance of *Legionella* and infected cells, but also reducing host cell availability for proliferation. Evidence supporting GM-CSF's role in controlling infections do exist, even with atypical pathogens such as *M. tuberculosis* (308–310).

Some of the cytokine responses observed in this study may be attributed to U937's M2-like expression profile (161,311). Cytokines with traditionally anti-inflammatory roles (TGF-β and IL-13) decreased while inflammatory markers (FGF-2 and GM-CSF) increased overall upon infection with clinical strains. This is at odds with the traditional anti-inflammatory role of M2 macrophages but may be explained by inflammation caused by infection. As expected, many of the activation and chemotactic factors also decreased upon infection. *Legionella* employs proteins that modulate host processes to create an intracellular niche for survival, with each species having a unique array of effector proteins (8,88,117). We believe that these distinct effector protein profiles are the primary cause of species-specific differences in host immune responses.

Two of the cytokines examined in this study are recognized by KEGG to be involved in the "Legionellosis pathway" (depicting the pathophysiology of Legionellosis), IL-8 and IL-12 (KEGG recognizes IL-12 as IL-12A and IL-12B) (312–314). While IL-8 was only observed in *L. pneumophila*-infected cells, IL-12p40 and IL-12p70 were both expressed at levels below their LOD, indicating that while they may be important, they are likely expressed by other cell types. IL-8 has also been noted to be relevant in several other *Legionella* studies, reinforcing the importance that it plays in LD (88,278,293,300,305).

Several of the differentially expressed cytokines seen in this study are members of the IL-17/Th17, phosphoinositide 3-kinase (PI3K), Raf/Ras, and JAK-STAT signaling pathways, indicating a high level of Th17 involvement and anti-viral responses in anti-*Legionella* immune responses (Table 8). JAK-STAT signaling, as seen through the decreases in GM-CSF and increases in IL-13, indicates the influence of extracellular signaling disruption in anti-*Legionella* responses in U937 cells (315).

FGF-2 and GM-CSF interact with the PI3K pathway, which is involved in processes relating to the cell cycle such as proliferation and death (Table 8). For intracellular pathogens, this can be manipulated to prolong

the life of a host cell for immune evasion and to siphon resources (316,317). FGF-2 and GM-CSF are also linked to Rab and Raf/Ras GTPases, respectively, which are known to be modulated by *Legionella* (137,318,319). The combination of *Legionella*'s GTPases that interact with host proteins and modulation factors controlling host GTPases create a vast landscape of virulence factors that remain to be studied. Thus, more studies need to be done on *Legionella*'s effector proteins, especially those in non-*L. pneumophila* species to better understand the pathogenesis and consequent host factor modulation.

While our study only looked at excreted cytokines, future studies should include immune-independent *Legionella*-induced host factor modulation in processes such as cell cycle, ubiquitination, vacuolar transit, and membrane production should be explored to understand *Legionella*'s virus-like pathogenesis further. Furthermore, studies can include quantitative real-time PCR to identify the downstream pathways impacted by the differentially regulated proteins.

The notable differences in cytokine responses induced by *L. pneumophila* Philadelphia-1 compared to the clinical species indicate that host responses to *Legionella* infections should be studied using clinical or more recently preserved strains. It is unclear if the differences seen between this study and others, and between other studies, are primarily due to strain differences or cell type.

In conclusion, we found that the replication capacity of non-*pneumophila Legionella* species can be comparable to that of *L. pneumophila*, suggesting that replication rate is not the sole reason they are infrequently recovered in clinical settings. Within the context of the strains used in this study, each species has different cytokines that are important in responses against their respective infection. Our cytokine findings, combined with findings from other researchers, suggest that host GTPases are a major target for *Legionella* pathogenesis.

Chapter 4: Final Discussion

Legionella causes community and hospital-acquired pneumonia that can be severe and is associated with mortality. As an environmental pathogen, *Legionella* is capable of causing common source outbreaks, and incidence appears to be increasing, with climate change implicated as one potential explanation (3,191,193,194,320). Despite the importance, most research focuses on *L. pneumophila*, while other groups of *Legionella* remain understudied, and in the absence of routine identification, pathogenesis is not well understood, and public health impacts are incompletely appreciated. This work discusses the gaps in global *Legionella* diagnostics and the implications of certain pathways being overrepresented in *Legionella*'s interactome with U937 cells through a cytokine study.

Our scoping review showed that diagnostic practices for *Legionella* should involve the use of molecular techniques such as PCR for enhanced sensitivity. While culture is frequently reported as being used in diagnoses, it has low sensitivity. Our findings that half of all cases of LD are caused by *Legionella pneumophila* serogroup 1 indicate that reliance on UAT as the sole diagnostic technique may miss half of all *Legionella* infections, a poor trade-off even for the rapidity of the test. A lesson to learn from our scoping review is that using a combination of techniques can provide a more thorough diagnosis covering a broader range of species or serogroups. Furthermore, using our search criteria, we were unable to find articles evaluating *Legionella* in immunocompromised populations, which could answer which immunological conditions have a higher association with specific species of *Legionella* to enhance diagnostic practices in individuals with certain conditions.

Improving *Legionella* diagnosis and monitoring is key for patient samples and environmental samples in water systems. An outbreak in the community and long-term care facilities in Ontario, Canada in 2022 was traced back to a cooling tower and resulted in 35 cases (29 hospitalized, 1 death) (321). While patient sample testing involved UAT and sputum culture, which was used in 5 cases with sputum samples, WGS and PCR were used to find the outbreak source. 27 cases occurred in individuals with unspecified chronic conditions, emphasizing the importance of studying key risk populations. A 2024 outbreak in London Ontario resulted in 30 cases, 9 ICU admissions, and 2 deaths, prompting the establishment of the *Legionella* investigation Reference Document from the Ontario Ministry of Health, which recommends culture and PCR testing of LRT specimens (322–324). Another outbreak in Argentina was also identified by sequencing BAL samples (65,325). These outbreaks underscore the need to incorporate PCR and WGS into diagnostics for quicker identification in outbreak responses. While it is unclear which chronic conditions were represented in the 2022 Ontario outbreak, it may be presumed that these conditions

were immunosuppressive. Altogether, there has been an increase in the incidence of *Legionella* and the culprits behind it are thought to be a global aging population, climate change, and aged and undermaintained water systems (3,4,11,32,326).

Moreover, *Legionella* pathogenesis and pathophysiology remain understudied, especially in non-*pneumophila Legionella* species, as many of the effector proteins remain unidentified. The representation of interference of cytokines involved in the JAK-STAT, PI3K, Raf/Ras, and IL-17/Th17 pathways found in our study suggests that common pathways are impacted in *Legionella* infections. However, no two species induced the same response, indicating that the mechanisms responsible are species-specific. Thus, the precise consequences and mechanisms imparted by *Legionella* on cellular processes such as ubiquitination, vesicular transport and nutrient uptake, signal transduction, and preventing apoptosis during infection are poorly understood (8,117,119,327). These pathways are important because *Legionella* manipulates them to create an intracellular niche.

Manipulation of ubiquitination allows *Legionella* to redirect host proteins or mark them for degradation to protect the LCV or even interfere with ubiquitination (260,327,328). Vesicular transport mechanisms are exploited to redirect macromolecules or prevent LCV acidification (327–332). Rab-family GTPases have been reported to be manipulated by a *Legionella* effector protein to transport endoplasmic reticulum-derived vesicles to the LCV while other GTPases have been reported to be involved in golgi fragmentation, influencing the fate of golgi body-derived vesicles (115,120,150,318,331). However, non-vesicular nutrient uptake methods are present in the form of MavN, a transmembrane transition metal transporter known to transport Fe^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+} (134,333). SidK has been reported to be involved in preventing LCV acidification (332).

Interference of signal transduction promotes *Legionella*'s survival by disrupting the immune response and also prevents apoptosis, allowing it to siphon nutrients from the host (106,327,328,334,335). *Legionella* has been reported to inhibit IFN-β signalling for its own survival through inhibition downstream of STAT1/STAT2 phosphorylation (175,334). While the precise mechanism for this is unclear, evidence supports a type 1 IFN pathway independent from IFN-γ in a mouse model (336).

Protection from *Legionella* infections has been reported to be dependent on cell-mediated immunity through Th1 responses, a subtype of CD4+ cells (327,337). Our study found decreased production of GM-CSF and IL-8, and increased production of anti-inflammatory markers TGF-β and IL-13, cytokines associated with the IL-17 pathway, which can affect Th1 responses (338). This finding is somewhat

corroborated by Tateda et al., who found increased levels of IFN-γ and IL-12 in serum from individuals with *Legionella* pneumonia, also emphasizing Th1 responses (337). HIV primarily eliminates CD4+ cells, which worsens the prognosis for individuals infected with *Legionella*. Imbalances in Th1 cells are also common in autoimmune diseases, either directly or through treatments, which increases susceptibility to infections (339,340). Thus, it is important to study the distribution of *Legionella* infections and efficacies of diagnostic practices in these populations.

While IL-4 was not included in our study, Newton et al. found that it plays a key role in facilitating Th1 responses in a humanized mouse model, increasing expression of IL-1β and TNF-α (278). However, this study also found increases in IL-6, which has been reported to induce Th1 differentiation, suppressing Th2 differentiation and contrarily do the opposite through suppressor of cytokine signaling (SOCS)-3 and SOCS-1, respectively (341,342). This suggests that IL-6 may indiscriminately induce Th differentiation, while the polarization depends on another factor.

In a study on plasma samples from adults living with HIV and CAP, Head et al. found that eotaxin, IP-10, and MCP-1 were higher in individuals co-infected with HIV and *Legionella* compared to other pathogens, and experienced worsened outcomes and increased ICU admission (250). The author notes that these are all markers of macrophage activation and differentiation (343–346). No overlap was found between the current study and Head et al.'s cytokines. However, all three of their cytokines are involved in IL-17 signaling, which is represented in the current study's findings (Table 8).

The main strength of our study is the compilation of works in the literature for evaluating the positivity rate of diagnostics available for non-*pneumophila Legionella* infections. This provides insights into diagnostic efficacy in *Legionella* infections globally with minimal species biases based on the current understanding of the landscape of LD etiology to inform future diagnostic practices. Another strength is the immunological characterization of the immune response caused by non-*pneumophila Legionella* species, which are infrequently studied. We used clinical strains rather than type strains to induce immune responses more reminiscent of natural infection.

A limitation of this study is that we have no background information on the bacterial strains obtained from the Health Sciences Centre used in the immunological study. How old the strains are and how many times they have been passaged is unclear, and thus mutations accumulated since isolation are unknown. This can influence the responses elicited by host cells during infection in ways that are not reflective of a natural infection.

Chapter 5: Conclusion

In conclusion, we have addressed gaps in knowledge in LD caused by non-*pneumophila Legionella* species by studying both the diagnostic and epidemiological landscape of LD, highlighting non-*pneumphila Legionella* species. Many *Legionella* infections are missed due to reliance on over-specific testing methods, especially those caused by non-*pneumophila Legionella*. This can be addressed by using a combination of methods accommodating for more diverse etiologies. We also studied immunological consequences of non-*pneumophila Legionella* infections in a macrophage-like cell model, finding similar responses occurring between etiologies, with species-specific differences, highlighting the distinct methods used to achieve the same outcome.

Chapter 6: Future Directions

The epidemiology of Legionella requires further investigation, as the exact geographical distribution of species and serogroups remains unclear. The clinically relevant species/serogroups of *Legionella* must be ascertained at the regional or national level across the globe and within specific populations such as PLHIV.

The differences in immunological pathology found in our infection studies and findings in the literature drive the next step in studies to look at species-specific differences in pathogenesis (8,118). This would be two-pronged, as studies need to examine both the role of host mechanisms and *Legionella* secretome in pathogenesis. Based on the findings of this study, the host factors to focus on would be those of the JAK/STAT, IL-17 pathway, PI3K, and Raf/Ras pathways for their occurrence in infection by all strains used in this study. Future work can build off of what was done here by examining the immune responses induced by non-*pneumophila Legionella* species in primary cell culture or more complex models such as air-liquid interface or cocultures involving multiple types of host cells.

Increasing concerns over antibiotic resistance warrant new methods to treat antibiotics. To circumvent the arduous process of drug development and approval, emphasis should be placed on repurposing approved drugs. Some work has been done in studying the effects of host-directed therapies on infections such as metformin (347–350). While host-directed therapies may see lower efficacy in extracellular infections, there is potential in using them to treat infections where the pathogen uses host factors for its survival such as in intracellular pathogens and viruses, making them a broad-spectrum treatment that can be applied before a full diagnosis. Furthermore, evidence exists for drugs that target secretion systems, which in the case of *Legionella* are crucial for intracellular survival. While most drugs have been designed to target T3SS, it is unclear how essential it is for *Legionella* survival (351–355). Thus, more drugs targeting T4SS need to be discovered or tested against *Legionella*.

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Chapter 8: Published Manuscripts During my Master's

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2. Ha R, Heilmann A, Lother SA, Turenne C, Alexander D, Keynan Y, Rueda ZV. The Adequacy of Current Legionnaires' Disease Diagnostic Practices in Capturing the Epidemiology of Clinically Relevant Legionella: A Scoping Review. Pathogens. 2024 Oct 1;13(10):857. doi: 10.3390/pathogens13100857. PMID: 39452728; PMCID: PMC11510479.

Chapter 9: Appendix

Appendix 1

*Protocol for *Legionella* infections in U937 cells obtained and modified from Brassinga Lab

ABBREVIATIONS:

BCYE	Buffered charcoal yeast extract
BSC	Biosafety cabinet
BYE	Buffered yeast extract
CT	Centrifuge (adjective)
FBS	Fetal bovine serum
Gen	Gentamycin
Gen100	100ug/ml gentamycin
HIBBS	Heat-inactivated fetal bovine serum
MOI	Multiplicity of infection
OD600	Optical density at 600nm, AKA Abs600
P100, P200, P1000, etc.	Pipet with the indicated volume in microliters
Pen strep, pen+strep, or PS	Penicillin-streptomycin mixture
PMA	Phorbol 12-myristate 13-acetate
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute

Materials and reagents

U937 maintenance

- RPMI1640 (Wisent, lot: 350007066)
- FBS (Wisent, lot: 115757)
- Penicillin Streptomycin (Sigma Aldrich, lot: 0000159238)
- PMA (Sigma Aldrich, lot: MKCT0606)

Legionella culture

- Agar (Wisent, lot:800010028)
- Bacto yeast extract (Gibco, lot: 2461100)
- Activated charcoal (Sigma Aldrich, source: 1003431134)
- α-ketoglutarate (Sigma Aldrich, source #: BCCH3270)
- Iron (III) pyrophosphate (Sigma Aldrich, source: SLCF7775)
- ACES (lot: SLCJ2815)
- L-cysteine (Wisent, lot: 550010EG)

U937 infection

- Trypan blue (Sigma Aldrich, lot: RNBK1828)
- Gentamycin (Sigma Aldrich, lot: 3697282)
- Molecular grade water (Wisent, lot: 809115110)

- PBS (Sigma Aldrich, lot: RNBM2022)
- Tween20 (Sigma Aldrich, lot: SLCK8931)

Equipment

- Biosafety cabinet
- Centrifuge
- Microscope (40x magnification)
- Hemocytometer
- Microcentrifuge
- Spectrophotometer
- CO2 incubator
- Water bath
- Pipet controller
- Serological pipets (5ml, 10ml, 25ml)
- 15mL conical tubes
- 50ml conical tubes
- Micropipettor (1ml, 200µl, 100µl, 20µl)
- Multichannel pipets (10µl, 100µl)
- Micropipet tips (1ml, 200µl, 100µl, 20µl)
- Microfuge tube (1ml)
- 24-well plate
- Breathable culture tubes
- Wooden skewers
- Shaking incubator with tube racks
- Petri dishes
- 96-well plate
- Tissue culture-treated T75 flasks

Procedure

<u>*Legionella* preparation</u>

1) *Legionella* species are grown on BCYE agar for 5 days at 37˚C supplemented with 5% CO_2
2) At 18-20 hours before infection, colonies are picked from *Legionella* plates and grown in BYE broth at 37˚C.

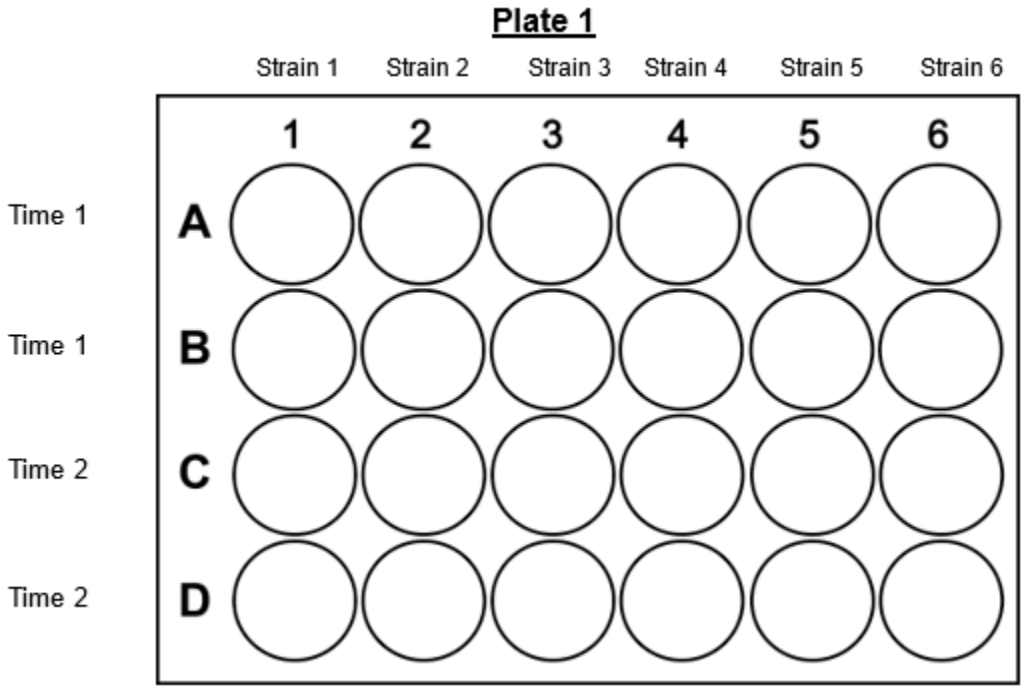
<u>U937 Differentiation</u>

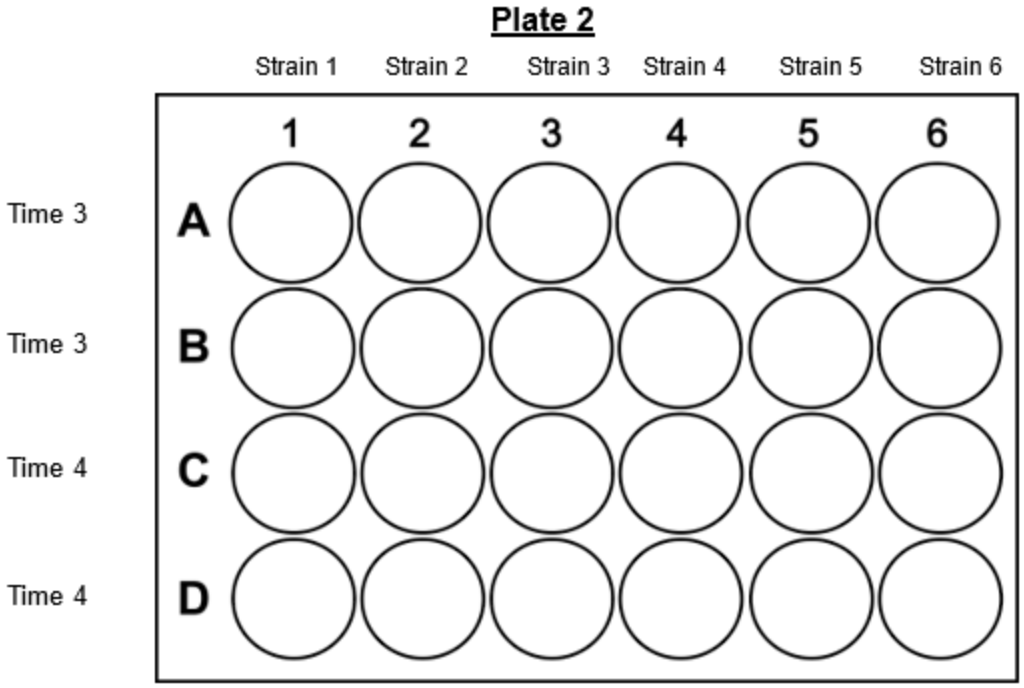
1) Remove a small amount of U937 cells from culture flask and transfer to a microfuge tube.
2) Transfer a portion of the removed U937 cells to another microfuge tube and dilute it 1:1 with trypan blue (need at least 20µl of U937/trypan blue mixture).
3) Using a hemocytometer, count U937 cells
4) Centrifuge enough U937 cells to achieve 1,000,000 cells/ml upon resuspension in 50ml of RPMI1640+10%FBS+1%penstrep. Centrifuge at 900RPM for 7 minutes.
5) Remove supernatant and resuspend U937 cells in a total volume of 50ml.

6) Add PMA to achieve a final concentration of 50ng/ml to the centrifuge tube and mix by inversion.
7) Add 1ml of the cell suspension to each well in a 24-well plate and incubate at 37˚C supplemented with 5% CO_2 for 24h on a shaking platform (overnight culture).

Infection (Use media without antibiotics)

1) Transfer 1ml of the overnight cultures to microfuge tubes and centrifuge them at max RPM for 1 minute.
2) Remove the supernatant and resuspend the culture in 1ml of RPMI1640+10%FBS
3) Prepare bacteria by checking OD_{600}. *Legionella*'s OD_{600} factor is 1.0 = $1x10^9$ cells/ml
4) Prepare a 10ml stock of bacteria in RPMI1640+10%FBS with a concentration of $2x10^6$ cells/ml to achieve a MOI of 2.
5) Wash each well of the 24-well plate with 1ml of fresh RPMI1640+10%FBS.
6) Remove the media from each well and add 1ml of the RPMI1640+10%FBS bacterial stocks to relevant wells.

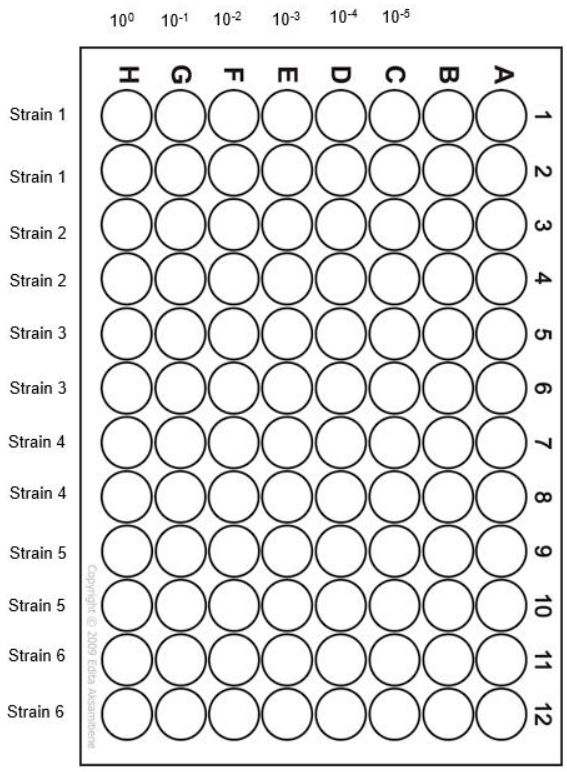




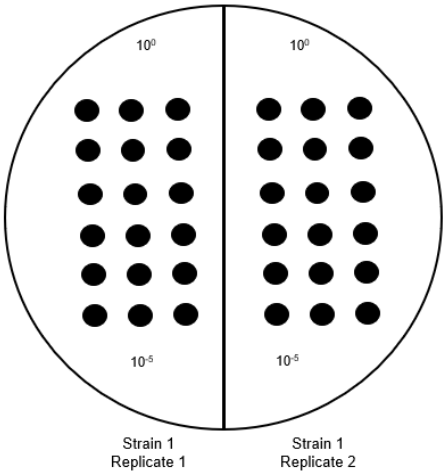
7) Incubate the plates for 1h at 37°C supplemented with 5% CO_2.
8) Prepare 500ml of 100 µg/ml gentamycin in RPMI1640+10%FBS.
9) After incubation, wash each well twice with fresh RPMI1640+10%FBS.
10) After the second wash, replace the media with the gentamycin supplemented media and incubate the plates for 1h at 37°C supplemented with 5% CO_2.
11) After incubation, wash each well thrice, leaving media in the wells.
12) After addition of media, incubate at 37°C supplemented with 5% CO_2 until desired timepoint.

Lysis and supernatant harvesting

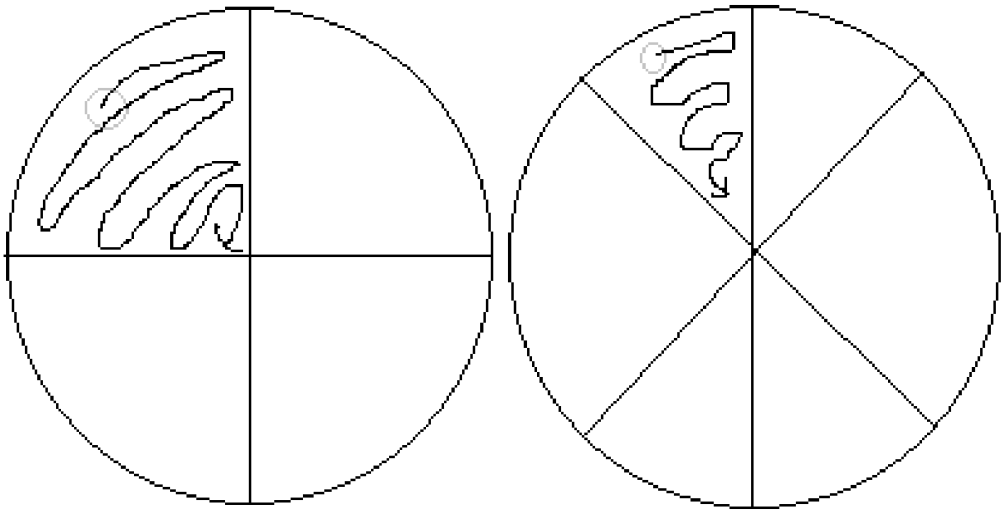
1) At desired timepoints, remove media from relevant wells and save them in microfuge tubes. Quickly add 1ml of 0.1% (v/v) Tween20-FBS to wells.
2) Lightly scrape the well bottoms with a 1ml micropipet tip. Let the plate sit for 25 mins.
3) Scrape the well bottoms again to ensure the monolayer is completely gone.
4) Transfer a few ml of molecular-grade water to a sterile petri dish
5) Using a 100µl set up a 96-well plate for serial dilutions if necessary by adding 90µl of molecular grade water to diluted wells (non-10^0 wells)



6) Transfer 100µl of lysates from the 24-well plate to the relevant 96-well plate wells.
7) If necessary, perform 10-fold dilutions by transferring 10µl from 10^0 wells to 10^{-1} wells and continuing onto 10^{-5}.
8) Return the plate into the incubator.
9) Plate 10µl from each dilution on BCYE agar according to one of the following formats in triplicate:



a. Spot plating



b. Drop 10µl of media onto a section of the plate. Bend the end of a pipet tip and use the folded part to spread the drop throughout the section of the plate

10) Incubate the plates at 37C supplemented with 5% CO_2 for approximately 4 days, until colonies are countable. Some species grow more slowly, so these can incubate an extra day or 2.

Supernatant preparation for cytokine analysis

1) Centrifuge the collected infection supernatants at 5000g/7200rpm for 5 mins in a microcentrifuge to pellet remaining bacteria
2) Transfer the resultant supernatants to new microfuge tubes (can aliquot if necessary), discarding the pellets. Store collected supernatants at -20˚C.

Recipes

FBS and penstrep supplemented RPMI1640

50ml of heat-inactivated FBS is added to a bottle (500ml) of RPMI1640. RPMI1640 should always be supplemented with FBS.

5mL of 10,000U penstrep is added to relevant bottles of RPMI1640-10%FBS.

BCYE agar (500ml)

Mix 5g of Bacto yeast extract, 0.5g of α-ketoglutaric acid, 0.5g of ACES buffer, 0.75g of activated carbon, 7.5g of agar, and 500ml of distilled water in a 1L flask. Adjust the pH to 6.6-6.7 with 6M KOH and autoclave. Once the media has cooled to ~55˚C, add 2ml of sterile 10% (w/v) cysteine and 0.5ml of sterile 25% (w/v) Iron III pyrophosphate. Plates can then be poured.

BYE broth (500ml)

Mix 5g of Bacto yeast extract, 0.5g of α-ketoglutaric acid, 0.5g of ACES buffer, and 500ml of distilled water in a 1L flask. Adjust the pH to 6.6-6.7 with 6M KOH and autoclave. Once the media has cooled to ~55˚C, add 2ml of sterile 10% (w/v) cysteine and 0.5ml of sterile 25% (w/v) Iron III pyrophosphate.

Note

- Cysteine must be prepared fresh each time media is made
- BCYE agar can be stored at 4˚C for 1 month
- BYE broth can be stored at 4C indefinitely without iron (III) pyrophosphate. Add iron pyrophosphate when you begin to use a specific bottle
- Iron III pyrophosphate is photosensitive. Cover in foil and store at 4˚C
- All work should be done in a sterile BSC if possible
- *Legionella* reaches early stationary phase at approximately 18-20h, where it is most infectious
- Ensure that cell culture media is pre-warmed to at least 25˚C before use
- To avoid cells drying out during infection, wash wells of 1 strain at a time