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**The title of my thesis  
which should be split on  
several lines if it is too long**

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[B]ecause we tend to reward others when they do well and punish them when they do badly, and because there is regression to the mean, it is part of the human condition that we are statistically punished for rewarding others and rewarded for punishing them — [Kahneman \(2012\)](#).



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## Preface

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First words and acknowledgements. And we add a lot of text to make sure that it spans more than one line, as otherwise it may not show up.



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## Abstract

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This example thesis briefly shows the main features of our thesis style, and how to use it for your purposes.



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# Contents

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<b>Preface</b>	<b>iii</b>
<b>Abstract</b>	<b>v</b>
<b>Contents</b>	<b>vii</b>
<b>List of Figures</b>	<b>ix</b>
<b>List of Tables</b>	<b>xi</b>
<b>List of Abbreviations and Notation</b>	<b>xiii</b>
<b>1 Introduction</b>	<b>1</b>
<b>2 Biological Background</b>	<b>7</b>
2.1 Microbial Host-Cell Infection . . . . .	7
2.1.1 Viral Infection Mechanisms . . . . .	8
2.1.2 Bacterial Entry Mechanisms . . . . .	10
2.1.3 Actin-based Intracellular Motility . . . . .	12
2.2 Select Bacterial Pathogens . . . . .	12
2.2.1 <i>Bartonella Henselae</i> . . . . .	12
2.2.2 <i>Brucella Abortus</i> . . . . .	14
2.2.3 <i>Listeria Monocytogenes</i> . . . . .	16
2.2.4 <i>Salmonella Typhimurium</i> . . . . .	18
2.2.5 <i>Shigella Flexneri</i> . . . . .	20
2.3 Select Viral Pathogens . . . . .	22
2.3.1 Adenovirus . . . . .	23
2.3.2 Rhinoviruses . . . . .	23
2.3.3 Vaccinia Virus . . . . .	23
2.4 RNA Interference . . . . .	23

## CONTENTS

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<b>3 Mathematical Background</b>	<b>25</b>
<b>A Complementary information</b>	<b>27</b>
A.1 Including R code with verbatim . . . . .	27
A.2 Including R code with the <i>listings</i> package . . . . .	28
A.3 Using Sweave to include R code (and more) in your report . . . . .	28
<b>B Yet another appendix....</b>	<b>29</b>
B.1 Description . . . . .	29
B.2 Tables . . . . .	29
<b>Bibliography</b>	<b>31</b>
<b>Epilogue</b>	<b>33</b>

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## List of Figures

---

1.1	Relative frequencies of death causes in 2012 by World Bank income groups . . . . .	3
1.2	Relative frequencies deadly infectious diseases for 2012 by World Bank income groups . . . . .	3
2.1	A generalized view of the steps necessary for viral–cellular membrane fusion . . . . .	9
2.2	Zipper and trigger mechanisms for bacterial host-cell entry . . . . .	11



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## List of Tables

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2.1	The Baltimore classification scheme for viruses . . . . .	10
B.1	Test results . . . . .	29



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## Abbreviations and Notation

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**AIDS** acquired immune deficiency syndrome.

**AMR** Antimicrobial resistance.

**Arp2/3** actin-related-protein 2 and 3.

**ATR** acid tolerance response.

**Cdc42** cell division control protein 42 homolog.

**COPII** coat protein complex II, involved in anterograde endoplasmic reticulum (ER)–Golgi transport.

**CSD** Cat Scratch Disease.

**ER** endoplasmic reticulum.

**ERAD** endoplasmic-reticulum-associated protein degradation.

**ERGIC** ER-Golgi intermediate compartment.

**GDP** guanosine diphosphate.

**GEF** guanine nucleotide exchange factors.

**GTP** guanosine triphosphate.

**HDT** Host directed therapeutics.

**HIV** human immunodeficiency virus.

**I(1,3,4,5,6)P<sub>5</sub>** inositol 1,3,4,5,6-pentakisphosphate.

**ICAM-1** intercellular adhesion molecule 1.

**Ics** *Shigella* intracellular spread protein.

**Ipa** *Shigella* invasion plasmid antigen.

**LPS** lipopolysaccharide.

## **LIST OF ABBREVIATIONS AND NOTATION**

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- M cells** microfold cells.
- N-WASP** neural Wiskott-Aldrich syndrome protein.
- PI(4,5)P<sub>2</sub>** phosphatidylinositol 4,5-bisphosphate.
- PI(5)P** phosphatidylinositol 5-phosphate.
- Rac1** Ras-related C3 botulinum toxin substrate 1.
- RhoG** Ras homology growth-related.
- SARS** severe acute respiratory syndrome.
- SCV** *Salmonella*-containing vacuole.
- SIF** *Salmonella*-induced filaments.
- Sip** *Salmonella* invasion protein.
- Sop** *Salmonella* outer protein.
- SptP** secreted effector protein.
- T3SS** type III secretion system.
- T4SS** type IV secretion system.
- TIR** toll-interleukin-1 receptor.
- Tir** translocated intimin receptor.
- TLR** toll-like receptor.
- VEGF** vascular endothelial growth factor.
- WHO** World Health Organization.

## Chapter 1

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# Introduction

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Infectious diseases have played an undeniably important role in human history. With human populations becoming sufficiently aggregated to sustain direct life cycle viral and bacterial infectants around 2000 BC, devastating invasions of a growing number of pathogens started to occur ([Dobson and Carper, 1996](#)).

One of the earliest well documented incidence of a large-scale epidemic is known as the Plague of Athens. Starting in 430 BC and lasting roughly three years, a highly infectious disease killed 75'000 to 100'000 people or 25% of Athen's population. This catastrophic event is attributed either to smallpox, a viral infection with *Variola major* or typhus, caused by *Rickettsia* bacteria ([Littman, 2009](#)).

The bacterium *Yersinia pestis* caused three major plague pandemics in the early and late middle ages, as well as in the late 19th century. Originating in northern Africa in 523 AD and spreading around the Mediterranean basin throughout the years 541–546, the Plague of Justinian is assumed to have killed up to half of the population of affected areas. The effect on cities was disproportionately severe. In Constantinople, for example, an estimated 230'000 people out of 375'000 lost their lives to the disease ([Treadgold, 1997](#)). Returning in the years 1347–1351, known today as the Black Death, a plague pandemic again wiped out around half of Europe's population. Death toll estimates range from 15 to 23.5 million ([Zietz and Dunkelberg, 2004](#)). Leaving behind a grim cultural heritage, this catastrophe had a lasting effect on economic and social structures in Europe. The third large-scale outbreak started around 1855 in southern China and quickly spread to Japan, Taiwan and India again wreaking havoc on the affected population.

Bringing diseases such as smallpox, measles (an infection with the *Measles virus*) and typhus to the Americas during the European invasion of the New World had grave repercussions for the indigenous population, carrying no

## 1. INTRODUCTION

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natural resistance towards the newly introduced pathogens. It is estimated that the population of present day Mexico fell from 20 million to 1.6 million over the course of the 16th century due to multiple disease epidemics, critically contributing to the successful colonization of the new continents ([Dobson and Carper, 1996](#)).

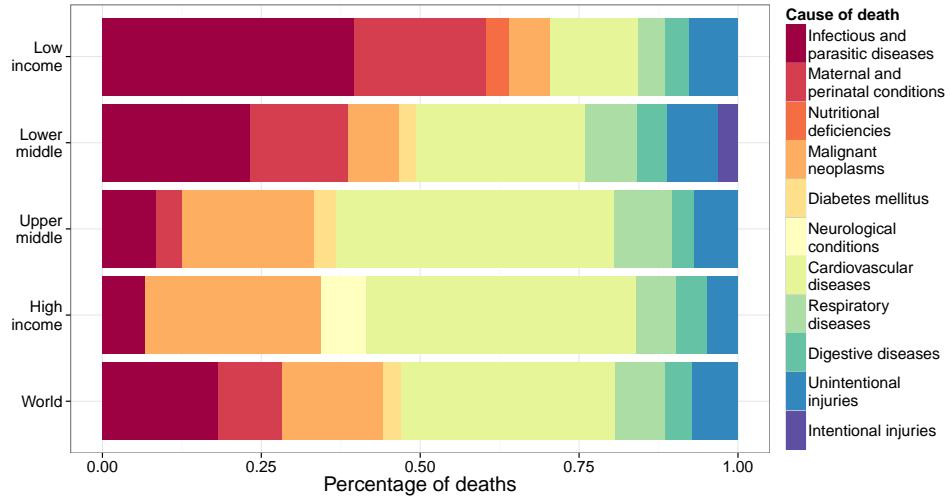
Cholera and influenza are further contagious diseases with high mortality rates, responsible for global epidemics. *Vibrio cholerae*, a bacterium which causes infections of the intestine, became widespread in the early 19th century and caused seven pandemics since, the last of which only started in 1961. Antibacterial treatment of sewage and purification of drinking water greatly help to prevent and contain spreading of the disease but in areas with inadequate sanitation, such as Haiti after the 2010 earthquake, it remains a pathogen difficult to control. The influenza virus causes seasonal epidemics characterized by low lethality rates among people with intact immune systems<sup>1</sup>. Irregularly occurring influenza pandemics, initiated by zoonosis of new virus strains, against which no natural immunity exists, however, are accompanied by much higher lethality rates. The most significant such event is known today as the Spanish flu pandemic of 1918, costing the lives of 50–100 million, nearly half of which were young, healthy adults ([Taubenberger and Morens, 2006](#)).

In addition to diseases plaguing humanity for centuries, new ones continually emerge. Human immunodeficiency virus (HIV) is believed to have transferred from non-human primates in the early 20th century and the recent outbreaks of severe acute respiratory syndrome (SARS) and swine flu serve as vivid illustrations of such occurrences.

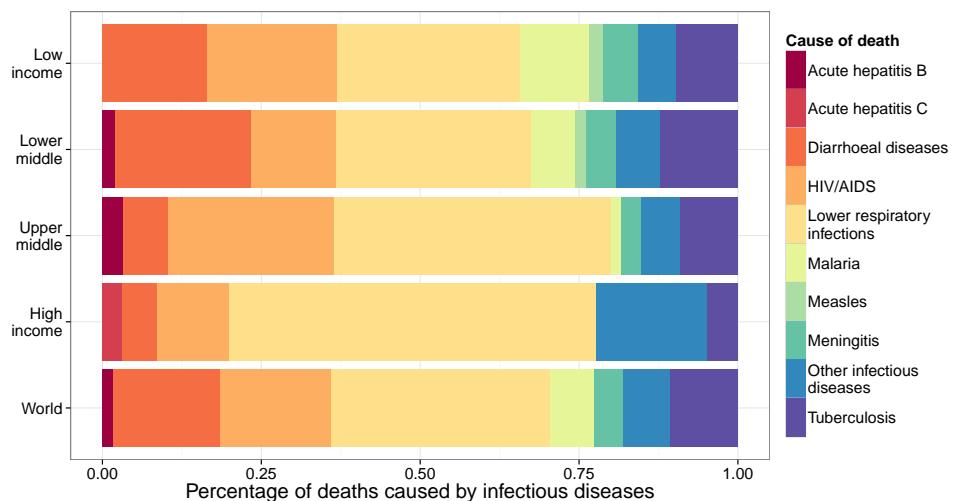
Despite development of means to treat and prevent many previously devastating diseases, infectious pathogens remain a serious threat to global health. In 2012, an estimated total of 58.3 million people died (20.1% in high, 29.4% in upper-middle, 36.5% in lower-middle and 14% in low income countries). Figure 1.1 partitions the total death count into World Bank income groups and causes. In low income countries, infective diseases are the most prevalent cause of death (39.6%), followed by maternal and perinatal complications with substantial margin (20.8%). In lower middle income countries, cardiovascular conditions catch up (26.5%), but are still almost matched in frequency by infectious diseases (23.3%). In upper middle (8.5%) and high income countries (6.7%), the importance of infectious disease while weakened remains accountable for a significant number of deaths. Globally, infectious diseases are the second most frequent cause of death (18.3%), even

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<sup>1</sup>In spite of low lethality, these seasonal epidemics still incur significant economic damages. The [World Health Organization \(2003\)](#) estimates annual health care costs and loss of productivity due to influenza at US \$71–176 billion for the United States of America alone.



**Figure 1.1:** Relative frequencies of death causes in 2012 by World Bank income groups. Binning is based on Gross National Income (GNI) per capita and the thresholds are \$1045 or less for low income, \$1046 to \$4125 for lower-middle, \$4126 to \$12745 for upper-middle and \$12746 or more for high income economies. The data was obtained from the [World Health Organization \(2012\)](#).



**Figure 1.2:** Relative frequencies of deadly infectious diseases for 2012 by World Bank income groups. Binning is based on Gross National Income (GNI; see figure 1.1). The data was obtained from the [World Health Organization \(2012\)](#).

more prevalent than all forms of cancer combined (15.8%) and only preceded by cardiovascular diseases (33.7%).

Focusing only on deaths caused by infectious disease, lower respiratory infections are most frequent (for each income region individually, low to high: 28.7%, 30.8%, 43.5% and 57.7% as well as worldwide: 34.5%; cf. figure 1.2). Diarrhoeal diseases and HIV/AIDS are the next most common worldwide

## 1. INTRODUCTION

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(16.9% and 17.3%, respectively) where diarrhea is more prevalent in lower income regions (16.6% and 21.4% versus 7% and 5.6%), while HIV/AIDS plays a major role irrespective of income region (low to high: 20.4%, 13.3%, 26.2% and 11.3%).

Dealing with highly virulent pathogens and preventing their spreading requires a multi-pronged approach. First and foremost, etiology and routes of transmission have to be understood. Knowledge of vectors and natural reservoirs is of great importance as a first line of defense. In the case of plague, for example, insecticides killing fleas were successfully used as a prophylactic measure, as well as controlling rat populations. Sanitary precautions including purification of drinking water, cooking foods well and the usage of disinfectants prevent initial infection, while measures such as sewage treatment, hand washing and wearing face masks help limiting spread among humans. Vaccination is the most important preventive measure. Exposing the immune system to a foreign antigen in a controlled manner artificially induces immunity. Among the great successes of widespread vaccination efforts is the global eradication of smallpox through a coordinated initiative lead by the World Health Organization in the 1970's.

Post-infection therapies include symptomatic treatments, as well as anti-infective drugs. Anti-bacterial or anti-viral agents exploit differences in proteomes between host and pathogen to selectively disable the invader with minimal toxicity to the host. This approach has been tremendously successful throughout most of the 20th century, leading to widespread application and prompting development of resistance towards the commonly used compounds. Adding to the severity of the problem is a lack of discovery of new drugs. No new class of anti-bacterial agents has been found since 1987, causing big pharmaceutical companies to withdraw from the area. The remaining research is mainly focused on improving on existing drugs, leading to a weak product pipeline, especially for the treatment of gram-negative bacteria ([Silver, 2011](#)). In its first global study on anti-microbial resistance, the [World Health Organization \(2014\)](#) notes:

Antimicrobial resistance (AMR) within a wide range of infectious agents is a growing public health threat of broad concern . . . A post-antibiotic era—in which common infections and minor injuries can kill—far from being an apocalyptic fantasy, is instead a very real possibility for the 21st century.

An alternative to pathogen directed search lies in targeting the set of host proteins necessary for infection. Many intracellular parasites subvert cellular functions to gain entry via host-mediated processes such as endocytosis. Upon entry, they move to a suitable niche and rely on host resources for proliferation. Challenges include evading host-cell defense mechanisms, generating sufficient space for replication, nutrient acquisition and keeping the

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host alive as long as possible, most of which require complex interactions between invader and host-based mechanisms. Finally, exiting the host cell again requires the parasite to successfully insert itself into existing signaling pathways ([Leirião et al., 2004](#)).

Host directed therapeutics (HDT) offer an escape from the conundrum of wanting to kill but not wanting to select for the surviving microbial parasites. The major challenge under this regimen is finding infectome components that are nonessential for cell survival, as orthogonality of host and infectant can no longer be exploited. Proving feasibility of the approach, [Czyż et al.](#) screened a library of 640 compounds already approved by the United States Food and Drug Administration (USFDA or FDA) for inducing resistance to four intracellular pathogens (*Coxiella burnetii*, *Legionella pneumophila*, *Brucella abortus*, and *Rickettsia conorii*). They found multiple drugs, not classified as antibiotics, that successfully inhibited intracellular bacterial growth while entailing only limited toxicity to host cells (THP-1). [Prussia et al.](#) review the usage of genome-wide screens to study host-pathogen interactions (for HIV and influenza) which in turn serve as basis for rational identification of drug targets for novel host-directed antivirals.

A detailed understanding of the human infectome is of crucial importance to the development of HDT and may even benefit the development of new anti-microbial agents. Feasibility of systematic loss of function screens using RNA interference methodology offers a unique opportunity to investigate complex cellular networks, making this an ideal tool for laying groundwork in combating infectious diseases. Of great importance, however, is ensuring reproducibility and comparability of such datasets, as well as ready availability to the scientific community.



## Chapter 2

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# Biological Background

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In order to better understand infectious diseases from a cell biological standpoint, this chapter reviews the current state of knowledge surrounding both bacterial and viral entry mechanisms. A sweeping overview of epidemiology and pathogenesis for several specific bacterial (*Bartonella henselae*, *Bacillus abortus*, *Listeria monocytogenes*, *Salmonella enterica* and *Shigella flexneri*), as well as viral parasites (*Adenovirus*, *Rhinovirus* and *Vaccinia virus*) is given and the chapter concludes with a look at RNA interference as this mechanism is a cornerstone of genome-wide knockdown experiments.

### 2.1 Microbial Host-Cell Infection

Multi-layered keratinized skin is impenetrable for almost all microbial parasites. Instead they either require breaches such as cuts, scratches, puncture wounds and arthropod bites, or environmental interfaces which offer less impervious protection. Examples include respiratory, gastrointestinal and urogenital tracts, which all contain segments where only a single layer of epithelial cells has to be overcome. Although often protected by chemical defense mechanisms (acidity of the stomach and urogenital tract, as well as microbicidal factors in mucous secretions in the respiratory tract and small intestine), combined with frequent flushing (urination, peristalsis and the coordinated beating of cilia), some microbes have adapted to survive these environments.

For extracellular pathogens to successfully colonize epithelial linings, they must avoid being removed by cleansing mechanisms of the host. Many bacteria accomplish this by expressing adhesins, protein complexes that recognize and bind to specific host-cell receptors, providing host and tissue tropism. Bacterial pili serve to extend reach and penetrate mucous secretions and therefore often carry adhesins. Enteropathogenic *Escherichia coli* have extended this scheme by injecting their own receptor protein Tir (translocated

## 2. BIOLOGICAL BACKGROUND

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intimin receptor) through the T3SS (type III secretion system) into the host cell to which it then attaches. This has the additional advantage that the intracellular domain of Tir can be used to modify host cell behavior ([Alberts et al., 2008](#)).

The outside of many epithelial barriers is covered in natural bacterial flora and crossing over into sterile cavities has the advantage of not having to compete with organisms well accustomed to that particular niche. Furthermore, intracellular pathogens are no longer accessible to antibodies and phagocytic cells and have a nutrient rich environment at their disposals. Mechanisms for entering host cells are described in the following sections.

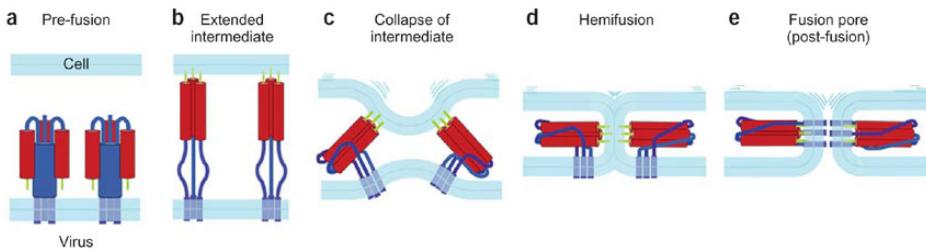
### 2.1.1 Viral Infection Mechanisms

Viruses are entirely dependent on host-cell metabolism and machinery for their replication, making them obligately intracellular. The first step of any entering sequence is binding to the target surface. This can be mediated by attachment factors which simply serve to concentrate the virions on the cell membrane or by virus receptors, which additionally act as communicators between host and pathogen. Common attachment factors include glycosaminoglycan chains and sialic acids and are comparatively unspecific. Glycoprotein spikes on enveloped and capsid proteins of non-enveloped viruses provide host specificity by binding cellular receptors. Mostly these cellular receptors serve other purposes which are exploited for infection. Binding affinity for individual interactions may be weak but aggregation of multiple interactions provide virtually irreversible avidity ([Smith, 2012](#)).

**Viral import.** For viral cell entry, different strategies exist. Enveloped viruses can either directly fuse with the plasma membrane (e.g. HIV) or be endocytosed by the host cell (e.g. influenza), while non-enveloped viruses either create a pore and directly inject their genome into the cytosol (e.g. poliovirus) or are endocytosed (e.g. adenovirus). Endocytosis has major advantages over alternative strategies. Reaching its replicatory niche within the host cell is a difficult task for a microorganism having no means of locomotion and hijacking the endocytic system solves this problem elegantly. Furthermore, maturation of endosomes provides precise environmental cues to the invader for triggering uncoating and release. Both fusion with the cell membrane and injection of viral material into the cytosol leaves back traces of infection to be detected by the immune system. Being completely engulfed by the host, virions leave back no telltale traces. Finally, lytic penetration techniques are not as problematic to the host if only applied inside an endosome.

Many endocytic viruses trigger clathrin-mediated, lipid-raft mediated or macropinocytic mechanisms. The clathrin pathway is most commonly used (for

## 2.1. Microbial Host-Cell Infection



**Figure 2.1:** A generalized view of the steps necessary for viral–cellular membrane fusion. (a) In pre-fusion conformation, fusion proteins have their hydrophobic fusion moieties tucked away. (b) Reacting to a trigger, such as low pH, the extended intermediate state is formed, characterized by exposed fusion sections, ready to interact with the target membrane. Conformational change in fusion proteins forces the two bilayers into close proximity, yielding first a collapsed intermediate state (c) and subsequently a state of hemifusion (d). Finally a fusion pore is formed (e), stabilized by the post-fusion conformation which is lower in energy than the pre-fusion state. ([Harrison, 2008](#))

example by rhinoviruses, some adenoviruses and influenza), while larger virions such as adenovirus 3 or vaccinia virus initiate macropinocytosis, an actin dependent formation of a lamellipodium which folds back onto the plasma membrane, creating a macropinosome. Lipid raft dependent pathways are poorly understood. Simian virus 40 and polyomaviruses initiate cholesterol dependent formation of lipid rafts and trigger endocytic uptake by inducing plasma membrane curvature.

Upon endocytic uptake, viral pathogens need to uncoat and eject their genetic material into the cytosol, as soon as their replicatory niche is reached. Escape timing is a critical issue, as late endosomes finally turn into lysosomes, capable of digesting their contents. Many enveloped viruses employ fusion mechanisms, which can be classified as type I or type II. For both types, increasing acidity associated with endosome maturation, initiates membrane fusion. Type I fusion proteins are forced into a metastable conformation prior to being added to the viral envelope and low pH triggers a conformational change to a state of lower energy. The energy released is used to force the two membranes close together resulting in their fusion (see figure 2.1). In type II fusion proteins, the critical transformation is not a conformational change but one in quaternary structure.

Non enveloped viruses cannot fuse with host membranes and have developed alternative approaches such as lysis (e.g. adenovirus) or ejecting their genome through pore-forming complexes (e.g. reovirus). Polyomaviruses need to pass through the ER because they rely on ER localized proteins to uncoat their capsid. For export from the ER into the cytosol, they exploit the endoplasmic-reticulum-associated protein degradation (ERAD) pathway, which serves as export mechanism for misfolded proteins from the endoplasmic reticulum to be degraded by proteasomes.

## 2. BIOLOGICAL BACKGROUND

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**Table 2.1:** The Baltimore classification scheme is based on diversity of genetic system that have evolved in viruses. For each group, a selection of virus families capable of infecting humans, is provided, along with whether the virions are enveloped and the location of their replicatory niche. The data was obtained from [Hulo et al. \(2011\)](#)

	Genome based class	Examples	Enveloped	Replication site
DNA viruses	Group I: dsDNA	Adenoviridae	no	nucleus
		Poxviridae	yes	cytoplasm
	Group II: ssDNA(+)	Parvovirinae	no	nucleus
		Anelloviridae	no	nucleus
RNA viruses	Group III: dsRNA	Reoviridae	no	cytoplasm
	Group IV: ssRNA(+)	Coronaviridae	yes	cytoplasm
		Picornaviridae	no	cytoplasm
		Hepeviridae	no	cytoplasm
	Group V: ssRNA(-)	Filoviridae	yes	cytoplasm
		Paramyxoviridae	yes	cytoplasm
Retro	Group VI: ssRNA(+)-RT	Orthoretrovirinae	yes	nucleus
	Group VII: dsDNA-RT	Hepadnaviridae	yes	nucleus

**Replication.** Requirements for replication of the viral genome, possible transcription and translation vary widely among different groups of viruses. A classification system, based on the diversity of mRNA production mechanisms has been devised by [Baltimore \(1971\)](#). Table 2.1.1 provides examples of human viruses for each of the 7 groups, whether those viruses are enveloped and where they replicate.

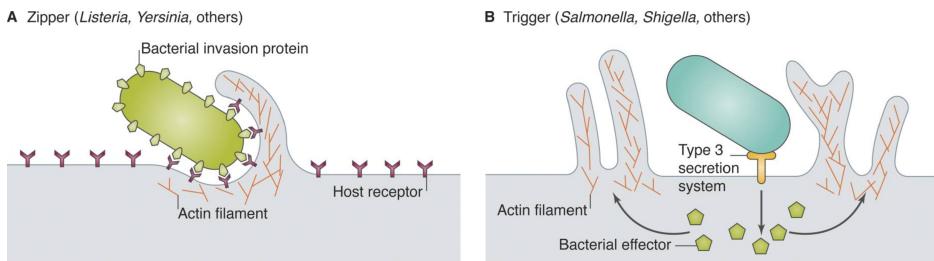
### Viral export.

#### 2.1.2 Bacterial Entry Mechanisms

Due to the much larger size of bacterial pathogens, endocytosis is not a feasible mechanism for entry. Phagocytosis, however can deal with particle uptake of this size magnitude. While phagocytosis is a function usually only available to macrophages, some bacteria have evolved mechanisms to induce phagocytosis in other cell types. Furthermore, species such as *Mycobacterium tuberculosis* and *Legionella pneumophila* targeting macrophages have to be able to escape phagosomes or deal with resisting digestion.

Two recurring patterns for inducing phagocytosis in non-phagocytic cells have been described: the zipper mechanism, found in *Yersinia pseudotuberculosis* or *Listeria monocytogenes* and the trigger mechanism used by *Salmonella enterica* and *Shigella flexneri*. Not all entry strategies can be assigned to these two classes and several additional, unrelated pathways have been described.

## 2.1. Microbial Host-Cell Infection



**Figure 2.2:** Both the zipper (A) and trigger mechanisms (B) are actin dependent and lead to phagocytosis by usually non-phagocytic host cells. Zippering bacteria display an invasion protein on their surface that recruits actin filaments via a host receptor, while triggering bacteria inject an effector into the host cytosol via a type III secretion system leading to uptake. ([Haglund and Welch, 2011](#))

**Zipper Mechanism.** The first step for zippering bacteria is binding to target cell receptors by expressing adhesins. *Y. pseudotuberculosis* displays invasins on its cell surface, capable of interacting with  $\beta_1$  integrins, while *L. monocytogenes* uses internalin A, a protein that binds E-cadherin. In both settings, a downstream signaling cascade leads to actin polymerization via recruitment of an Arp2/3 (actin-related-protein 2 and 3) complex and Rac1 (Ras-related C3 botulinum toxin substrate 1), yielding a phagocytic cup.

Cadherin and integrins are usually involved in anchoring cell junctions and the host cell is fooled into thinking a neighboring cell is initiating formation of such a junction. Responding by recruiting actin at the site of bacterial attachment to cover the surface of mistaken invasion proteins leads to engulfment and phagocytosis to the bacterium. This process incurs only modest cytoskeletal rearrangements.

**Trigger Mechanism.** In case of the trigger mechanism, bacterial T3SS weakly adhere to target cell receptors (CD44 for *Shigella*) and effector molecules are injected into the host cytosol through a pore formed by Sip (*Salmonella* invasion protein) or Ipa (*Shigella* invasion plasmid antigen) proteins. These induce major actin rearrangements that result in localized ruffling of the plasma membrane and subsequent swallowing of the bacterium.

Early in *Shigella* entry, VirA, secreted through the T3SS causes local destabilization of microtubules by binding to  $\alpha/\beta$ -tubulin heterodimers. This in turn stimulates Rac1 activity, Cdc42 (cell division control protein 42 homolog) recruitment and subsequent Arp2/3 activation leading to protrusions formed by actin filaments. IpaC recruits the Src tyrosine kinase further enhancing actin dynamics. Upon closing of the phagocytic pocket, the T3SS-secreted protein IpaA binds to vinculin and induces actin depolymerization.

*Salmonella* inject the proteins Sop (*Salmonella* outer protein)E and SptP (secreted effector protein), an activator/inhibitor pair for the GTPase complex

## 2. BIOLOGICAL BACKGROUND

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Rac1/Cdc42, into the host cell. First, guanine nucleotide exchange factors (GEF) activity of SopE induces massive actin-rearrangements leading to membrane ruffling and facilitating phagocytosis, followed by GAP activity of SptP, restoring the inactive guanosine diphosphate (GDP) state of Rac1/Cdc42 and leading to actin depolymerization. SopE is degraded more rapidly than SptP, enabling the invading pathogen to reversibly control the pathways exploited for entering.

**Other Entry Pathways.** In addition to trigger and zipper type uptake, other atypical mechanisms exist. Host cell entry of *Brucella abortus*, for example, has been described as invasome mediated ([Dehio, 2005](#)). In this actin dependent process, bacteria aggregate on the cell surface and trigger their engulfment by injecting bacterial effectors into the host via type IV secretion system (T4SS). The internalized structure is called an invasome. Actin-independent uptake albeit rare, is possible as evidenced by *Campylobacter jejuni*, which have evolved a microtubule dependent invasion strategy ([Kopecko et al., 2001](#)).

### 2.1.3 Actin-based Intracellular Motility

## 2.2 Select Bacterial Pathogens

A total of 5 bacterial pathogens were selected for study within the InfectX RTD project by SystemsX. This section shortly describes each organism in terms of microbiological features, pathogenesis, epidemiology and diseases caused in humans. For each organism, a chapter of [Rolain and Raoult \(2006\)](#) serves as basis and is augmented by one or two review article referenced in the first paragraph of each section.

### 2.2.1 *Bartonella Henselae*

*Bartonella henselae* is a short, rod shaped, unflagellated proteobacterium, phylogenetically closely related to the genus *Brucella*, presenting 94.4% 16S rRNA gene sequence homology, compared with *Brucella abortus*. The Gram-negative bacillus is a facultative anaerobic, intracellular parasite and was first described in 1992. Relatively harmless for healthy humans, infections can become life threatening in immunocompromised patients, making the species an important opportunistic pathogen ([Anderson and Neuman, 1997](#)).

**Diseases.** In immunocompetent humans, infection with *B. henselae* can lead to a condition known as Cat Scratch Disease (CSD). As the name suggests, most patient report being in contact with a cat and transmission often occurs through scratches and bites. Affecting primarily children and young adults (80% are 21 or younger), the self limiting infection typically presents

## 2.2. Select Bacterial Pathogens

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itself with lymphadenopathy. Most patients remain afebrile and do not report feeling ill, with low-grade fever and malaise shown in roughly 30% of the cases. Recovery from uncomplicated CSD usually takes 2 to 6 months and requires no specific treatment.

Possible complications include Parinaud's oculoglandular syndrome (granulomatous conjunctivitis in one eye and parotid lymphadenitis on the same side), splenomegaly and hepatic or splenic abscesses, accompanied by fever, weight loss, fatigue and malaise. In 1 to 7% of the cases, the disease spreads to the central nervous system, leading to encephalopathy, but recovery is usually rapid (within several weeks).

Infections with *B. henselae* tend to have more severe consequences for immunocompromised patients, such as bacillary angiomatosis, bacteremia and endocarditis. Acquired immune deficiency syndrome (AIDS) patients suffering from CSD usually experience severe, progressive disease with infection spreading systematically and without appropriate treatment, fatal outcome. *Bartonella spp.* are the only prokaryotes known to be able to induce angiogenic tumors such as bacillary angiomas, which may involve skin, respiratory or gastrointestinal epithelia, heart, liver, spleen, bone marrow, muscles, or lymph nodes. Bacteremia may lead to inflamed heart valves, usually requiring endocarditic patients to have heart valve replacement surgery.

**Pathogenesis.** *B. henselae* are capable of intracellular growth in both epithelial cells, and erythrocytes. Bundle forming type IV pili are essential for binding to target cells, making them important virulence factors. Internalization into red blood cells may be spectrin mediated and the bacterial protein deformin may also be involved. Endothelial receptors involved include intercellular adhesion molecule 1 (ICAM-1) uptake is either via endocytosis or by a unique cellular structure termed an invasome.

Vasculogenesis is induced though an increased production in vascular endothelial growth factor (VEGF) by infected host cells. Currently it is poorly understood how the pathogen provokes overexpression but the mechanism has been determined to be protease sensitive.

**Epidemiology.** The role of cats and in particular kittens, as reservoirs to *B. henselae* has been firmly established. Infected felines are asymptomatic and show no signs of illness. Cat fleas (*Ctenocephalides felis*) serve as vectors to spread the bacteria among cats and have also been suspected of infecting humans. The main path of transmission to humans however, is through scratches and bites by infected cats. *B. henselae* has also been found in ticks and tick bites prior to contraction of CSD have been reported.

In the United States, 24000 cases of CSD are reported yearly, yielding 2000 hospital admissions with an estimated health care cost of \$12 million. Chil-

## 2. BIOLOGICAL BACKGROUND

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dren are more likely to be affected (80%) and incidence is higher in males (60%). The seasonal pattern (occurrences higher in fall/winter) is attributed to cat mating patterns, as well as pet acquisition fluctuations.

### 2.2.2 *Brucella Abortus*

The Danish physician David Bang first isolated *Brucella abortus* in 1895 from cyetic cattle tissue, investigating a contagious disease causing abortions in cows. *B. abortus* are small, unflagellated proteobacteria with a cell wall consisting of an outer layer of lipopolysaccharide (LPS) (9 nm) and an inner layer of muramyl mucopeptide complexes (3–5 nm). The Gram-negative coccobacilli appear to have evolved from free-living, soil-dwelling species and are closely related to other human pathogens such as *Bartonella* spp., based on 16S rRNA sequences. *Brucella* species were investigated for possible use as warfare agents in the mid 20th century by several armed forces. ([Atluri et al., 2011](#))

**Diseases.** Brucellosis is a human disease caused by several pathogenic *Brucella* species, most importantly *B. abortus*, *B. melitensis*, *B. canis* and *B. suis*. Onset may be acute or insidious and due to protean symptoms, diagnosis based on clinical presentation alone is difficult. The febrile disease is generalized and may involve many parts of the body, including nervous, skeletal, gastrointestinal, cardiovascular, respiratory and genitourinary systems. Furthermore, as the bacteria spread to other reservoir hosts via their reproductive systems, persistence of infection is crucial to the pathogen and it comes as no surprise that brucellosis can manifest as a chronic disease in humans too.

Fever is the most consistent sign of *Brucella* infection and depending on what specific organs are affected, further symptoms include asthenia, anorexia, nausea, malaise, arthritis, hepatomegaly, splenomegaly, epididymo-orchitis in males, and pulmonary manifestations such as bronchitis or pneumonia. A rare complication (less than 2%), albeit the most lethal, is infective endocarditis. Invasion of the nervous system occurs in less than 5% of cases and often results in meningitis or meningoencephalitis with good prognosis under antimicrobial treatment.

**Pathogenesis.** Host entry happens primarily via the digestive system but is also possible through the respiratory tract or skin lesions. On the gastrointestinal route, *Brucella* spp. target Peyer's patches (lymphoid nodules localized towards the end of the small intestine) and must therefore pass through acidic conditions in the stomach. This is facilitated by expression of two ureases capable of hydrolyzing urea and producing a protective bicarbonate buffering system. When entering through the respiratory system,

## 2.2. Select Bacterial Pathogens

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*B. abortus* target alveolar macrophages which serve as access point to the lymphatic system therefore facilitating systematic spread.

In order to persist at systemic sites, both active and passive mechanisms for evading the immune system are in place. LPS of the outer cell wall disguises the bacteria from toll-like receptors(TLRs) and expression of two proteins containing toll-interleukin-1 receptor (TIR) domains actively interferes with TLR signaling.

Uptake by macrophages happens via phagocytosis, which is either triggered by nonopsonized bacteria through a lipid raft mediated mechanism or by opsonisation. Although opsonin marked bacteria are 10-fold more likely to be ingested, the number of pathogens reaching their replicatory niche within the host cell is higher for nonopsonized bacteria. Maturation of early endosomes into lysosomes is important for successful infection as preventing acidification (through addition of bafilomycin A) or fusion with lysosomes (through suppression of the late-endosomal GTPase Rab7) interferes with bacterial replication. Nonopsonized bacteria finally become associated with rough ER and begin replication in ER derived vacuoles within the ER-Golgi intermediate compartment (ERGIC). Blocking the small GTPase Sar1 inhibits intracellular replication by preventing acquisition of coat protein complex II, involved in anterograde ER–Golgi transport (COPII) by ER-exit vesicles and the small GTPase Rab2, involved in ER–cis-Golgi traffic, is required for maximal proliferation.

Despite multiplying intra-cellularly in high numbers, host cells are kept alive and are even able to replicate despite infection. Furthermore *Brucella* species are able to interfere with apoptosis, maintaining their replicatory niche, protected from immune response.

**Epidemiology.** Preferred natural reservoir species for *B. abortus* are cattle (*Bos taurus* and *Bos indicus*) and almost all parts of the world are affected. The disease exists in both domestic and wild animals and is most prevalent in Mediterranean countries, North Africa, throughout the Middle East, India, Central Asia, as well as South and Central America. Zoonosis most often occurs through ingestion of unpasteurized milk products but airborne transmission is also possible, putting professionals involved in animal husbandry at risk. Vertical transmission among reservoir hosts can occur through lactation and horizontal transmission is facilitated by mating and placental discharge associated with aborted gestation. Human-to-human transmission is rare (but has been suspected to be possible via sexual intercourse), making humans dead-end hosts. As opposed to *Bartonella*, immunodeficient patients do not seem to be especially susceptible towards *Brucella* infections.

Worldwide, an estimated 500000 new cases of brucellosis occur annually, making it one of the most prevalent zoonoses. Although usually susceptible

## 2. BIOLOGICAL BACKGROUND

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to combined antibiotic therapies of at least two agents (usually a tetracycline antibiotic combined with an aminoglycoside or rifampin), untreated brucellosis leads to a high degree of morbidity, leading to being classified a neglected zoonosis by the World Health Organization (WHO).

### 2.2.3 *Listeria Monocytogenes*

The short, Gram-positive bacilli are non-sporeforming facultative anaerobes, capable of growing in a wide temperature range (0–50°C) and in many different environments. Flagellation is temperature dependent with flagellin being expressed and assembled into peritrichous flagella around 20–25°C but not at 37°C. First described in 1924 by Murray after isolation from lymph glands of diseased laboratory animals, the pathogen was found to also infect humans four years later. For much of the time since, listeriosis was considered a rare zoonotic disease and did not receive much attention. It was not until the 1980s, when several food-borne listeria outbreaks caused a shift in interest towards the pathogen, which has since become a well studied facultatively intracellular parasite ([Farber and Peterkin, 1991](#)).

**Diseases.** Maternal and neonatal listeriosis accounts for almost half of all infections. Listeriosis in pregnancy typically manifests in bacteraemia and presents as a self-limiting febrile disease with flu-like symptoms. Many cases, however are asymptomatic and the first sign of infection is abortion or neonatal listeriosis. Maternal infection does not necessarily carry over to the fetus, especially if proper chemotherapy is administered. Perinatal incidences are divided into early and late onset (>5 days after parturition), with former cases typically resulting in septicemia and latter cases in meningitis. While in early onset cases the predominant route of transmission is transplacental, the situation is less clear in late onset cases. Both the maternal genital tract during child birth and environmental sources have been implicated. Despite antibiotic treatment, overall mortality rates of 30–40% are typical and prognosis for early onset disease is worse, as it is often associated with preterm birth and advanced stage of infection.

Among adults, most cases of listeriosis occur in T-cell deficient individuals. HIV infection, for example, increases incidence 150–300 fold compared to general population control groups. Predisposing conditions include lymphoreticular neoplasms, deliberate immunosuppression (e.g. antirejection treatment after organ transplants), alcoholism and diabetes mellitus. Despite increased susceptibility caused by immunodeficiency, roughly 30% of all infections affect immunocompetent individuals. In healthy adults, consumption of food contaminated with *L. Monocytogenes* can either lead to self-limiting febrile gastroenteritis with short incubation time (<24 h) or invasive listeriosis with much longer incubation periods (3–4 weeks). The systemic

## 2.2. Select Bacterial Pathogens

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form of infection often manifests as bacteraemia or as a neurological infection, but can also involve endocarditis and spread to other parts of the body. Central nervous system involvement occurs in as much as 75% of cases and either presents as meningitis or encephalitis. Mortality rates of 35–45% have been reported for listeriosis in adults.

**Pathogenesis.** The predominant entry path for *L. Monocytogenes* into the human body is via the gastrointestinal tract, where Peyer's patches are targeted. The bacteria can induce cellular uptake (see section 2.1.2), even by non-phagocytic host cells via expression of cell-surface associated interanlins. Upon cell entry, the phagosomal membrane is lysed, mediated by haemolysin secretion, and actin polymerization (see section 2.1.3) provides means of intracellular movement. Listerial haemolysin (listeriolysin) is acid activated with an optimum around pH 5.5, which is reached in late endosomes and membrane lysis is caused by the formation of large transmembrane pores. Growth and replication occur in the cytoplasm and adjacent cells can be entered by pushing against the plasma membrane and forming a pseudopod-like structure which in turn is taken up the neighbor. The resulting double-membrane vacuole can be escaped by cytolysis.

In an extracellular setting, haemolysins serve to rupture erythrocytes in order to generate free iron, a limiting growth factor. Furthermore the non-specific immune system has to be evaded and haemolysins have also been shown to be cytotoxic towards leukocytes. Additionally, expression of bacterial superoxide dismutase mitigates the effect of free superoxide radicals which play an important role in killing phagocytized bacteria.

**Epidemiology.** Incidence of listeriosis has initially been increasing since its recognition as food-borne disease but effects of awareness and diagnostic methods are unclear. While typically long incubation periods do pose difficulties for clinical diagnosis, the number of susceptible individuals is on the rise and certain aspects modern processing and handling of foods may be beneficial for growth of *L. Monocytogenes*. Disease rates of 2–15 cases per million population per year have been reported and listeriosis is among the leading case of lethal food-borne pathogen infections. Most recent data, however suggests, that incidence is decreasing again.

Due to its non-fastidious lifestyle, *L. Monocytogenes* has been isolated from a wide array of ecological niches, including soil, sewage and water (both fresh water bodies and estuaries). High persistence (up to 4 years) in soil samples is problematic when contaminated manure is used as fertilizer and biofilm formation poses challenges for eradication from food processing plants. Additionally, the ability to grow in refrigerated foods and resistance towards heat treatment such as pasteurization warrant alertness and special preventive care. Many different foods have been implicated in listeriosis outbreaks,

## 2. BIOLOGICAL BACKGROUND

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including vegetables (potatoes, radishes and celery), seafood (shrimp, crab-meat and smoked fish), dairy products (soft cheese, pasteurized and unpasteurized milk) and meats (poultry, various types of sausages and pâté).

Despite high prevalence in food (studies have found 20–80% of meat product samples and 1–10% of dairy product samples contaminated with *L. Monocytogenes*), comparatively few successful infections occur. The bacteria are ingested frequently in small doses and stool sample examinations suggest that 10-70% of investigated populations could be intestinal carriers. While the minimum infectious dose has not been settled definitively, approximations range from  $10^6$  to  $10^9$  bacteria.

### 2.2.4 *Salmonella* Typhimurium

*Salmonella* are non-sporulating, Gram-negative bacilli, belonging to the family Enterobacteriaceae. The motile bacteria are able to produce peritrichous flagella and diameters span 0.7 to 1.5 µm while typical lengths range from 2 to 5 µm. They are closely related to the genus *Escherichia*, showing only 15% chromosomal sequence disparity. Currently, two distinct species, *S. bongori* and *S. enterica*, within the genus *Salmonella* are recognized, both of which are pathogenic towards a wide array of hosts. *S. enterica* is further divided into 6 subspecies, the most relevant of which for human and domestic animal hosts being *enterica*. A large number of serovars (more than 2500) for *S. enterica* subsp. *enterica* have been characterized and due to an originally mistaken classification as separate species, some serovars are designated with shortened names. *S. Typhimurium*, therefore is shorthand for *S. enterica* subsp. *enterica*, serovar Typhimurium and to emphasize that Typhimurium is not a species description it is not italicized.

The first description of the genus *Salmonella* dates back to an investigation into swine fever led by Salmon and Smith in 1885. The newly isolated bacterium was wrongly proposed as the etiological agent, as the disease later turned out to be caused by a virus ([Fàbrega and Vila, 2013](#)).

**Diseases.** Two distinct disease patterns are associated with *Salmonella* spp. infections, typhoid fever and salmonellosis. While the former is exclusively caused by the serovars Typhi and Paratyphi, the latter is associated with several serovars, the most frequent being Enteritidis and Typhimurium, accounting for 65% and 12% of cases worldwide. The current and following sections will not discuss typhoid fever.

Salmonellosis is a diarrheal disease with a short incubation period of 6–24 h, followed by nausea, vomiting, loose or liquid bowel movements, abdominal cramps and fever. Clinical features are similar to those of dysentery and other gastroenteric disease and can include bloody and mucosal stool. In

## 2.2. Select Bacterial Pathogens

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most cases, the infection is self-limiting and symptoms fade away within 4 to 7 days. The most common complication is bacteraemia, which presents in 5% of cases and is more likely to develop in children, especially if malnourished, and immunocompromised individuals. Further manifestations of invasive infections include meningitis, osteomyelitis, cholangitis, pneumonia and endocarditis. While mortality in immunocompetent hosts in developed countries is as low as 0.1%, it can increase to 77% for HIV positive patients in undeveloped countries.

**Pathogenesis.** In order to reach the small intestine, ingested *S. Typhimurium* first are exposed to the hostile environment of the stomach. A set of proteins, summarized as acid tolerance response (ATR) helps mediate the acidic conditions and improves survival rates. The reamining bacteria subsequently reacht the small bowel and target its epithelial cells, with preference towards microfold cells (M cells). Flagellar motility enables penetration of intestinal mucus secretions and improves the chance of reaching the intestinal walls where adhesion can be established. Fimbriae are important attachment factors, capable of interaction with host-cell laminin and fibronectin and provide an initial platform for pathogen induced phagocytosis via trigger mechanism (see section 2.1.2).

*S. Typhimurium* utilize two separate T3SS systems for host-cell colonization, the first of which (T3SS-1) mediates invasion. In addition to strengthening initial interactions attaching the pathogen to its target, the needle like structure serves as delivery mechanisms, capable of injecting effector proteins, including SopE, SopE2, SopB (also known as SigD) and SipA. SopE serves as GEF and activates Rac1 and Cdc42 via GDP/GTP exchange, which in turn initiate cytoskeletal rearrangements. SopE2 is a further GEF, highly homologous to SopE and it is assumed to fill in for SopE in *Salmonella* strains missing the SopE gene. SopB is a phosphatase, capable of hydrolyzing various substrates, including inositol 1,3,4,5,6-pentakisphosphate ( $I(1,3,4,5,6)P_5$ ), which has been linked to intestinal fluid secretion causing diarrhea and phosphatidylinositol 4,5-bisphosphate ( $PI(4,5)P_2$ ), which is involved with membrane rigidity. Finally, the actin binding protein SipA stimulates actin polymerization and promotes membrane ruffling.

Upon engulfment, maturation of the phagosome and fusion with lysosomes have to be prevented. Unlike other pathogens that escape the digestive mechanisms of phagocytic vesicles by moving to the cytosol, *Salmonella* replicate inside phagosome-derived *Salmonella*-containing vacuoles(SCVs). In this setting, the second translocation complex (T3SS-2) is activated, and used to secrete effectors, including SsaB and SifA, capable of interacting with vesicular trafficking mechanisms and guiding the SCV away from its original degradation pathway. Furthermore T3SS-2 translocated effectors induce actin polymerization events, which guide the SCV toward a perinuclear position. A

## 2. BIOLOGICAL BACKGROUND

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last step in creating the intracellular niche needed for replication, is formation of *Salmonella*-induced filamentss(SIFs), extending outwards from the SCV. T3SS-2 secretes effectors such as SifA, PipB2, SseF and SseG, which mediate the microtubule dependent processes by bundling and accumulating microtubules and regulating microtubule motor function.

**Epidemiology.** The global disease burden caused by nontyphoidal salmonellosis is estimated at 90 million cases per year and 150000 deaths. Incidence rates are highest in East and Southeast Asia (up to 4000 cases per 100000 population per year) and both developed and undeveloped countries are affected (incidence rates in Africa are estimated at 320 while estimates for Europe are around 690 cases per 100000 population per year). An estimated 80.3 million or 86% of reported cases are food borne ([Majowicz et al., 2010](#)).

In order to control *Salmonella* outbreaks, preventive measures in food production and processing is of major importance. This starts with disease containment in domestic animals, such as vaccination of chickens, enforcing hygiene standards in manufacturing and distribution facilities and ends with proper preparation, exploiting heat sensitivity of the organisms. As the main route of transmission is fecal-oral, good sanitary infrastructure, treatment of sewage and water processing are crucial prerequisites in combating outbreaks of salmonellosis.

### 2.2.5 *Shigella Flexneri*

*Shigellae* are small, non-sporeforming, Gram-negative bacilli and belong to the family *Enterobacteriaceae*, along with *Escherichia*, *Salmonella* and *Yersinia*. While flagellar genes are present and their expression is observed under certain conditions, the bacteria are usually described as non-motile and unflagellated. The facultative intracellular parasite shows strong specificity towards human hosts where it typically infects the lower gastrointestinal tract.

*Shigella dysenteriae* was identified as the etiological agent of dysentery by Shiga in 1897 during an epidemic in Japan with 91000 reported cases and a >20% mortality rate. *S. Flexneri* was first described by Flexner in 1900, while investigating diseases endemic to the Philippines. Recent genetic studies suggest, that *Shigella* spp. belong to the species *Escherichia coli*, rather than forming a distinct genus, as only marginal sequence divergence (1.5%) between *E. coli* and *S. Flexneri* was found ([Schroeder and Hilbi, 2008](#)).

**Diseases.** Bacillary dysentery is an acute infection of the intestine. Mild cases of the disease are self-limiting and afebrile with diarrhea and possibly vomiting as the only symptoms. In as little as 24 hours after onset, bowel movements usually begin to normalize and the condition is resolved within

## 2.2. Select Bacterial Pathogens

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a few days. More severe cases are accompanied by strong abdominal cramps, fever and watery diarrhea containing blood and mucus, indicative of injury to the intestinal epithelium. While still usually self limiting in healthy individuals, recovery takes 10–14 days and relapses are possible. In immunocompromised patients, young children, especially if malnourished, and elderly individuals, life threatening complications including bacteraemia, renal failure, intestinal perforation and toxic megacolon are more frequent. Involvement of the central nervous system and respiratory tract is rare.

Administration of antibiotics is not recommended in mild to moderate cases as the disease can usually be overcome by the immune system and AMR in *Shigellae* is becoming an increasing concern. Oral rehydration therapy is the most effective treatment, helping the body replenish liquids and salts lost due to diarrhea. For severe infections, the use of antibiotics can become necessary and testing for resistance patterns, if possible, is advised.

**Pathogenesis.** Main targets of *S. Flexneri* are mucosa of the distal ileum and colon where they enter epithelial cells from the basolateral side. For initial crossing over from the apical side, action of M cells at Peyer's patches is exploited. These specialized enterocytes constantly sample antigens from the gut lumen and pass them to intraepithelially located dendritic cells and lymphocytes. Upon uptake by basolaterally located macrophages, *S. Flexneri* survive digestive action of the phagosomal vacuole by disrupting the surrounding membrane and initiating host-cell apoptosis, mediated by the bacterial effector protein IpaB, capable of acting on a caspase 1 regulated apoptotic pathway. The bacteria are subsequently released into the sub-mucosa, where they induce phagocytosis by normal epithelial cells via trigger mechanism (see section 2.1.2).

Initial contact between pathogen and target host cell is mediated by cellular  $\alpha_5\beta_1$  integrins and binding of IpaB to CD44 receptors may initiate first actin rearrangements, preparing the cell for uptake. Subsequent injection of at least 6 effector proteins into the epithelial cytosol through the T3SS triggers engulfment of the bacterium in an actin dependent process, involving the small GTPases Rac1 and Cdc42, which recruit the actin nucleation complex Arp2/3. IpaC, a component of the translocation complex, is involved in stimulating Rac1 and Cdc42 GTPase activity through an unknown mechanism and the secreted effector proteins IpgB1, IpgB2, IpgD and IpaA facilitate actin polymerization. IpgD, an inositol phosphate phosphatase, catalyzes the hydrolysis of PI(4,5)P<sub>2</sub> to PI(5)P which promotes disassociation of cytoskeleton and membrane, increasing actin availability and making the plasma membrane more susceptibility to manipulation. The mechanism of action of IpgB2 remains to be resolved, while IpgB1 is assumed to mimic activated RhoG (Ras homology growth-related), a small GTPase, located upstream in the signaling pathway of Rac1. Finally, binding of IpaA to vin-

## 2. BIOLOGICAL BACKGROUND

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culin induces depolymerization of actin, which is assumed to be important for closing of the phagocytic cup.

The resulting phagocytic vacuole has to be escaped before maturation progresses, which is accomplished in a non-acid dependent fashion, by the translocator proteins IpaB, IpaC and IpaD via membrane lysis. With release into the epithelial cytosol, the replicatory niche of *S. Flexneri* is reached. Actin mediated intracellular motility enables intercellular dissemination (see section 2.1.3) and targeting of epithelial tight junctions initiates break-down of the epithelial barrier, providing more pathogens with access to the basolateral side of the gut lining.

Host-cell actin utilization, is driven by activity of two bacterial proteins. VirA is secreted at the forward facing end of the rod shaped bacilli, which promotes degradation of tubulin structures and therefore clearing a path through the dense network of microtubules and surface bound Ics (*Shigella* intracellular spread protein)A facilitates actin polymerization at the opposite end. Both Arp2/3 and neural Wiskott-Aldrich syndrome protein (N-WASP) are involved in actin nucleation, the directed nature of which provides the driving force for locomotion.

**Epidemiology.** Estimates by the WHO peg the disease burden causey by *Shigella* spp. at 80 million annual cases worldwide, leading to 700000 deaths. Developing countries are disproportionately affected, representing 99% of all cases, as are children less than 5 years old, accounting for 70% of cases and 60% of deaths. In developed countries, incidence rates of 1–2 per 100000 population are typical and *Shigellae* are common causes of Traveller's diarrhea.

The predominant route of transmission is fecal–oral, highlighting the importance of sanitary precautions for infection control. Proper treatment of fecal matter is important for preventing contamination of drinking water and inhibiting spreading by disease vectors such as house flies. During acute phases, diseased individuals excrete pathogens in large numbers and as few as 100–200 organisms are sufficient of causing a new infection.

## 2.3 Select Viral Pathogens

In addition to the previous 5 bacterial pathogens, 3 viruses were selected for study within the InfectX RTD project by SystemsX. This section shortly describes each pathogen in terms of physical features, pathogenesis, epidemiology and diseases caused in humans. For each section, a chapter of [Craighead \(2000\)](#) serves as basis.

### 2.3.1 Adenovirus

Diseases.

Pathogenesis.

Epidemiology.

### 2.3.2 Rhinoviruses

Diseases.

Pathogenesis.

Epidemiology.

### 2.3.3 Vaccinia Virus

Diseases.

Pathogenesis.

Epidemiology.

## 2.4 RNA Interference



## Chapter 3

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# Mathematical Background

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Modeling the relationship among variables is one of the most important applications of statistical theory. The study of regression analysis (and the closely related notion of correlation) started to form towards the end of the 19th century with Sir Francis Galton's study of height heredity in humans and his observation of regression towards the mean. Over the next few years, Udny Yule and Karl Pearson cast the developed concepts into precise mathematical formulation, in turn building on work performed by Adrien-Marie Legendre and Carl Friedrich Gauss who developed the method of least squares almost a century earlier ([Allen, 1997](#)).

A multiple linear regression model can be written in matrix-vector form as

$$y = X\beta + \varepsilon \quad (3.1)$$

where  $y \in \mathbb{R}^n$  is the vector of observations on the dependent variable, the design matrix  $X \in \mathbb{R}^{n \times p}$  contains data on the independent variables,  $\beta \in \mathbb{R}^p$  is the  $p$ -dimensional parameter vector and the error term  $\varepsilon \in \mathbb{R}^n$  captures effects not modeled by the regressors. Without loss of generality, all variables are assumed to be expressed as deviations from their means and measured on the same scale.

In order to find unknown coefficients  $\beta_i$ , the ordinary least squares estimator minimizes the residual sum of squares, the squared differences between observed responses and their predictions according to the linear model.

$$\hat{\beta} = \arg \min_{\beta} \|y - X\beta\|^2 \quad (3.2a)$$

$$= (X^T X)^{-1} X^T y \quad (3.2b)$$

Some assumptions are typically associated with linear regression models that yield desirable attributes for the estimates. None of these restrictions are imposed on the explanatory variables; they can be continuous or discrete

### 3. MATHEMATICAL BACKGROUND

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and combined as well as transformed arbitrarily. Furthermore, in practice, it is irrelevant whether the covariates are treated as random variables or as deterministic constants. With exception of the field of econometrics it appears that the majority of literature adheres to the latter interpretation and therefore, statements will not explicitly be conditional on covariate values.

**Linearity.** The relationship between dependent and independent variables should be linear (after suitable transformations) and individual effects additive. If this cannot be satisfied, a linear model is not suitable.

**Full rank.** For the matrix  $X^T X$  to be invertible, it has to have full rank  $p$ . Therefore  $n \leq p$  and all covariates must be linearly independent.

**Exogeneity.** All independent variables should be known exactly i.e. contain no measurement or observation errors as only the mean squared error of the dependent variable is minimized. Additionally, all important causal factors have to be included in the model. Exogeneity implies  $E[\varepsilon_i] = 0 \ \forall i$ , as well no correlation between regressors and error terms (Hayashi, 2000).

**Spherical errors.** This includes both homoscedasticity or constant error variance:  $E[\varepsilon_i^2] = \sigma^2 \ \forall i$  and uncorrelated errors  $E[\varepsilon_i \varepsilon_j] = 0 \ \forall i \neq j$ . These two conditions can be written more compactly as  $\text{Var}[\varepsilon] = \sigma^2 I_{n \times n}$ .

**Normality.** For the estimated coefficients to gain some additional desirable characteristics, it can be required that the errors  $\varepsilon_i$  be jointly normally distributed. Together with the above restrictions on expectation and variance, this yields  $\varepsilon \sim \mathcal{N}_n(0, \sigma^2 I_{n \times n})$ .

Violations of these assumptions have varying consequences. In case of perfect multicollinearity, the ordinary least squares estimator  $\hat{\beta}$  as defined in (3.2b) does not exist. Recovering such a situation is possible by using a generalized matrix inverse (for example the Moore–Penrose pseudoinverse) or employing a regularization scheme such as ridge regression.

Omitting a variable that is both correlated with dependent variables and has an effect on the response (a nonzero true coefficient) will introduce bias in the parameters. The method of instrumental variables can help to produce an unbiased estimator.

The assumption of spherical errors ensures that the least squares estimator is the best linear unbiased estimator in the sense that it has minimal variance among all linear unbiased estimators. Heteroscedasticity and autocorrelation do not cause coefficient estimates to be biased but can introduce bias in OLS estimates of variance, causing inaccurate standard errors. A generalized least squares estimator (for example weighted least squares)

## Appendix A

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# Complementary information

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Additional material. For example long mathematical derivations could be given in the appendix. Or you could include part of your code that is needed in printed form. You can add several Appendices to your thesis (as you can include several chapters in the main part of your work).

### A.1 Including R code with verbatim

A simple (rather too simple, see [A.2](#)) way to include code or *R* output is to use `verbatim`. It just prints the text however it is (including all spaces, “strange” symbols,...) in a slightly different font.

```
## loading packages
library(RBGL)
library(Rgraphviz)
library(boot)

## global variables
X_MAX <- 150
```

This allows me to put as many spaces as I want.  
I can also use \ and ' and & and all the rest that is usually only accepted in the math mode.

I can also make as  
many  
line  
breaks as  
I want... and  
where I want.

## A.2 Including R code with the *listings* package

However, it is much nicer to use the *listings* package to include R code in your report. It allows you to number the lines, color the comments differently than the code, and so on.

```
1 ## example to generate an .eps file with the function ps.latex()
2 ## Author: Sarah Gerster and Martin Maechler
3 ## Last revision: 16 Aug 2011
4
5 require("sfsmisc") # pdf.latex(), pdf.end(), etc
6
7 pdf.latex(file='test_plot.pdf') #, main=TRUE)
8 ## no main=TRUE is needed to leave enough space for the plot title
9 ## but see below
10
11 ## make sure the legends are large enough
12 par(cex=1.5)
13
14 ## Make sure your lines are "visible" enough. Otherwise your plot
15 ## won't look very nicely in your text.
16 plot(-10:10, (-10:10)**2, type="l", lty=5,
17       xlab="my_x", ylab="my_y",
18       ## no main title: NOT recommended for figures in text which
19       ## have a \caption{...}
20       lwd=4, col='blue')
21 lines(-10:10, 0:20, type="p", lwd=4, pch=23, col='red')
22 legend(-3, 90, c("func1", "func2"), lwd=4, col=c('blue', 'red'),
23         lty=c(1,1), cex=1)
24 pdf.end() # starts the previewer (which refreshes itself;
25           # at least on Linux at SfS
```

## A.3 Using Sweave to include R code (and more) in your report

The easiest (and most elegant) way to include R code and its output (and have all your figures up to date with your report) is to use Sweave. You can find an introduction Sweave in /u/sfs/StatSoftDoc/Sweave/Sweave-tutorial.pdf.

## Appendix B

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# Yet another appendix....

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### B.1 Description

**Something** details.

**Something else** other definition.

### B.2 Tables

Refer to Table B.1 to see a left justified table with caption on top.

Table B.1: Results.	
Student	Grade
Marie	6
Alain	5.5
Josette	4.5
Pierre	5



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## Epilogue

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A few final words. Test 2



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