In vitro and *in vivo* virulence of *Salmonella* Typhimurium DT104: a parallelogram approach

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Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. M.J. Kropff,
in het openbaar te verdedigen
op donderdag 5 juni 2008
des morgens te elf uur in de Aula

Berk, Petra Angeli
In vitro and *in vivo* virulence of *Salmonella* Typhimurium DT104 – 2008

Thesis Wageningen University, Wageningen, The Netherlands – with summary in Dutch – 168 p.

Keywords: *Salmonella* Typhimurium DT104 / acid resistance / virulence / parallelogram approach

ISBN: 978-90-8504-934-0

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ABSTRACT

In 1984 the first *S.* Typhimurium DT104 isolate resistant to five antibiotics: ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (R-type ACSSuT) was detected. Since then this multi-antibiotic resistant strain has become epidemic in the United States, Canada and different countries in Europe, including the Netherlands. In the Netherlands in 2001 of all *S.* Typhimurium isolated from humans, 37.2 % were DT104 of which 80.8 % was resistant to 5 or more antibiotics. Some studies have shown that the clinical symptoms of multi-drug resistant *S.* Typhimurium DT104 are more severe than other *S.* Typhimurium or *S.* Enteritidis infections and, that due to their antibiotic resistance, these infections are difficult to treat.

The aim of this thesis is to investigate the effect of environmental factors (most importantly acid) on the virulence of *S.* Typhimurium DT104. Also the relation between *in vitro* intestinal cell experiments is determined to *in vivo* tests using an animal model to enable a prediction for *S.* Typhimurium DT104 virulence in humans.

Since the stomach with its acidic pH is recognized as the first line of defence of the human body against food borne pathogens, acid resistance of pathogens may contribute to their virulence. The acid resistance of 37 *S.* Typhimurium DT104 strains, isolated from both food and patients, was determined. A large variation was found in the level of acid resistance. Interestingly, all high acid resistant strains were isolated from patients suggesting a positive correlation between acid resistance and virulence.

Five *S.* Typhimurium DT104 strains (one reference strain, two acid resistant and two acid sensitive strains) were selected and used for virulence testing in cell lines *in vitro* and in a rat model *in vivo*, by measuring their infectious capacity. The human intestinal cell line Caco-2 was used to determine different infectivity parameters (adhesion, invasion and the induction of the immune response). Irrespective of the growth pH (5 or 7) all five *S.* Typhimurium DT104 strains showed similar adhesive and invasive capacity. Also the induction of the immune response (IL-8 production) was similar for all strains. However *S.* Typhimurium DT104 strains grown to the exponential phase showed increased invasion but not adhesion and IL-8 production, compared to the same strains grown to stationary phase. In a rat intestinal cell line comparable results were found as with the human intestinal cell line.

In vivo a rat model was used to study the infectivity of three different *S.* Typhimurium DT104 strains (one reference strain, one acid resistant and one acid sensitive). The rat

model appeared to be a reproducible model. There is a clear dose-response relationship for the total white blood cell population on day 6 after infection. Also microbiological counts in different parts of the intestine and the spleen showed a clear dose-response relation. As was found *in vitro* no significant effect on the *in vivo* infectivity was found between the different *S.* Typhimurium DT104 strains. Also growth at low pH had no significant effect on the *in vivo* infectivity. Furthermore no significant differences in infectivity were found when oral doses were administered with or without bicarbonate to neutralize the gastric acid. However growth phase had a large effect on the *in vivo* infectivity. The ID_{50} (the dose at which 50 % of the animals is infected) was 30-60 times lower for strains grown to the exponential phase than that of strains grown to the stationary phase.

Using both *in vitro* and *in vivo* results, a parallelogram approach was used to determine the probability of one *Salmonella* infecting the host. In this approach data derived from different *in vitro* cell systems (both human and rat cell lines) can be compared with each other and data from experiments with animal cells can be compared with *in vivo* animal data. Extrapolation can now follow two routes: from *in vivo* animal data to man and from *in vitro* human data to man. Both routes of the parallelogram approach were used to determine the probability of one bacterium causing an infection in humans. This parameter together with the dose was used to determine the probability of infection in humans. *Salmonella* strains grown to the exponential phase have a probability of infection for humans that is about 30-60 times higher than that of bacteria grown to the stationary phase. Acid resistant strains however have a probability of infection for humans that is almost 100.000 times higher than that of acid sensitive bacteria. It can be concluded that growth phase, and more importantly, acid resistance are important determinants for the virulence of *S.* Typhimurium DT104.

CHAPTER 1

Introduction

P.A. Berk

INTRODUCTION

Non-Typhoidial salmonellosis is the name for the acute lower gastrointestinal tract disease caused by infection by *Salmonella*. Salmonellosis is generally contracted through the consumption of contaminated food of animal origin (mainly meat, poultry, eggs and milk products), although many other foods, including vegetables contaminated from manure, have been implicated in its transmission.
The clinical course of human salmonellosis is usually characterized by onset of fever, abdominal pain, diarrhoea, nausea and sometimes vomiting. A clinical case usually lasts several days but the clinical signs and disease course are variable, depending on host factors, bacterial strain, food product and dose. Infected people (and animals) often shed the *Salmonella* in their faeces for many days. A small portion become chronic carriers and shed sporadically for months or years. Although *Salmonella* is usually restricted to the intestinal tract, invasive forms of the infection can reach the bloodstream and cause bacteraemia with affecting other body organs and cavities with severe consequences, sometimes without the presence of gastrointestinal signs. Bacteriaemia is more severe and the risk of death is the highest in the very young, the elderly, the pregnant and the immunocompromised. In addition, a small percent of cases in healthy individuals are complicated by chronic reactive arthritis. Generally the disease is self limiting and allowed to run its course rather than treat the patient with antibiotics that may damage the tissues or select for antibiotic resistant bacteria. Dehydration occurs in some patients and fluid replacement may be necessary. Diagnosis usually consists of isolating *Salmonella* from stool specimens or rectal swabs. Like other bacterial food-borne infections, clinical cases are more common during the summer than other seasons. Although outbreaks usually attract media attention, studies indicated that more than 80% of all salmonellosis cases occur individually rather than as outbreaks.

INTERNATIONAL OUTBREAK OF SALMONELLA TYPHIMURIUM DT104

Sweden, (Eurosurveillance Weekly 2001;5 (29): 010719)
In early June 2001, at least 10 people resident in the south of **Sweden** were found to be infected with ***Salmonella* Typhimurium definitive phage type (DT) 104**. They were mostly children with a predominance of Arabic names. The first case fell ill on 13 April and the latest reported case on 19 June. The investigation showed that **27** people had become infected after consuming **helva**, a type of dessert or sweet made from sesame seeds. *Salmonella* from the same type has also been directly isolated from five jars of helva, four with pistachio and one with cocoa flavouring.

Australia, (Eurosurveillance Weekly 2001;5 (32): 010809)
The **Australian** health authorities had been investigating a cluster of **14 cases** of ***S.* Typhimurium DT104** in Melbourne, Victoria and had been unable to identify the vehicle of infection. The information disseminated through Enter-net allowed them to review the cases and identify a clear epidemiological association with **helva** imported from Turkey.

Germany, (Eurosurveillance Weekly 2001;5 (33): 010816)
Various foods containing sesame seeds from retail and delicatesse stores in the state were sampled and microbiologically examined. Besides confirming the known contamination of selected **helva** products, ***S.* Typhimurium DT104** was also isolated from a **sesame paste** manufactured by the same producer. There is a strong association between illness caused by *S.* Typhimurium and a Turkish or Arabic first and last name in 2001, compared with the same period in 2000.

United Kingdom (Eurosurveillance Weekly 2001;5 (33): 010816)
Local sampling is being undertaken by the Public Health Laboratory Service and the local authority environmental health departments to determine whether any of these products currently on sale in **England** and **Wales** are contaminated with *Salmonella* spp. One sample of **helva** has been found positive for ***S.* Typhimurium DT104**.

Norway, (Eurosurveillance Weekly 2001;5 (33): 010816)
Norway's Statens Institutt for Folkehelse has observed an unusually high number of infections caused by ***Salmonella* Typhimurium definitive phage type 104** since November 2000. The **29 cases** have occurred in 24 apparently independent incidents. Turkish or Arabic surnames are prominent among the cases.

SALMONELLA TYPHIMURIUM DT104

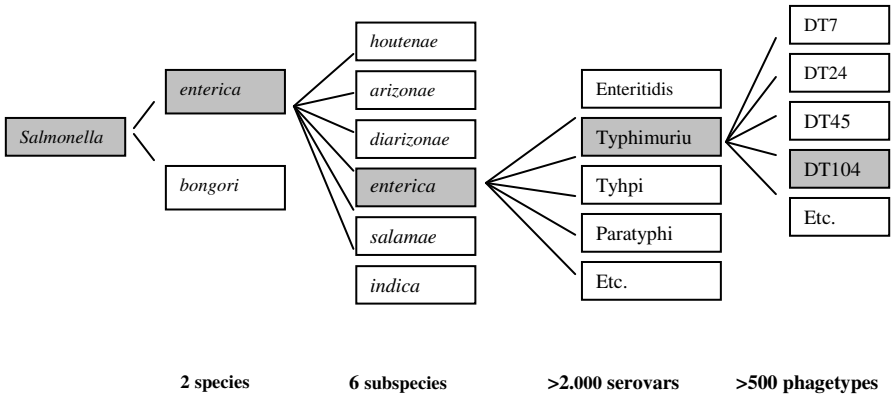


Figure 1.1 Schematic representation of salmonellae classification and diversity[1].

Salmonella is a member of the *Enterobacteriaceae* family. The first strain of *Salmonella* was discovered and reported in 1885 by D.E. Salmon, a veterinary surgeon. Today there are more than 2400 known serotypes, grouped in the two species *bongori* and *enterica* Fig. 1.1). The last is divided in six subspecies including *enterica* (subspecies I), to which serovar Typhimurium belongs. The formal scientific name for this organism is *Salmonella enterica* subspecies *enterica* serovar Typhimurium, shortly *S.* Typhimurium. *Salmonella* is sensitive for bacteriophages, but not all *Salmonella* strains are sensitive to the same phages. Therefore, by using the sensitivity for different phages *S.* Typhimurium can be divided further. The emerging *S.* Typhimurium phage type DT104 is one such group of *S.* Typhimurium. The first known *S.* Typhimurium DT104 isolate was identified in Canada in 1970. In 1984 the first *S.* Typhimurium DT104 isolate resistant to five antibiotics: ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (R-type ACSSuT) was detected [2]. The number of cases with multi-resistant *S.* Typhimurium DT104 rose from 259 in 1990 to 406 in 1996 in the United Kingdom [3]. Since then the multi-antibiotic resistant strain has become epidemic also in other countries in Europe, the United States and Canada [4]. Also in the Netherlands the percentage of isolates with multiresistant *S.* Typhimurium DT104 increased rapidly until 2001[5] (Figure 1.2). In the Netherlands, at the beginning of this study, in 2001, of all *S.* Typhimurium isolates from humans, 43 % were DT104 of which 80.8 % was resistant to five or more antibiotics. Molecular studies have demonstrated that the resistance genes are chromosomally encoded. This is important because removal of the selective pressure from antibiotics is not expected to reverse resistance, as it might with extrachromosomal mediated resistance.

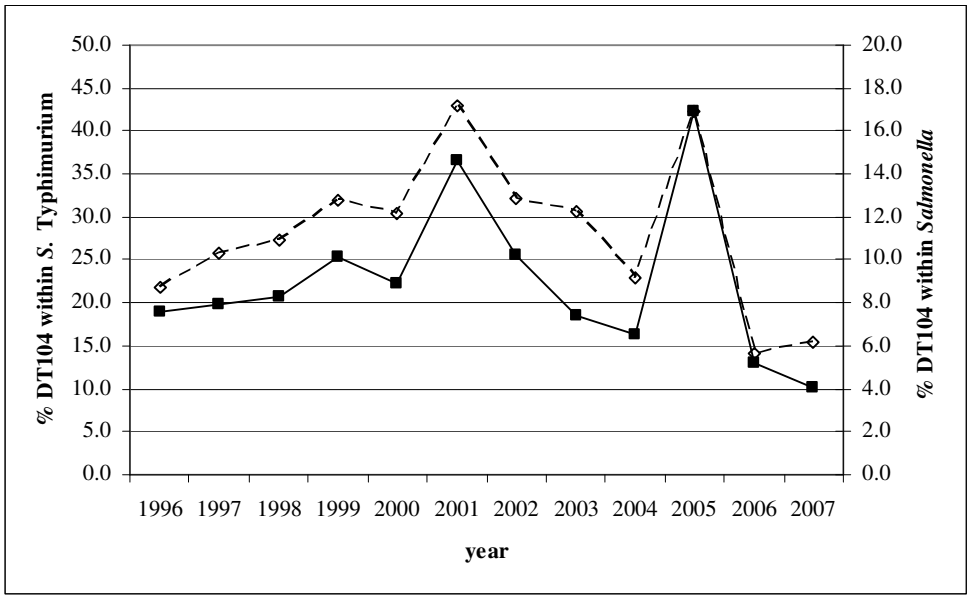


Figure 1.2. Percentage of* S. *Typhimurium DT104 isolates from all* Salmonella *isolates (■) and from* S. *Typhimurium isolates (◊) from patients. Used with permission of W. van Pelt.

Some studies have shown that the clinical symptoms of multi-drug resistant *S.* Typhimurium DT104 are more severe than other *S.* Typhimurium or *S.* Enteritidis infections [6;7] and, due to their antibiotic resistance, difficult to treat. Several studies have indicated that infection with *Salmonellae* resistant to one or more antimicrobial drugs is associated with increased risk for hospitalization, invasive illness and death [6;8-13].
Thus *S.* Typhimurium DT104:

1 is an emerging pathogen,
2 is persistently multi antibiotic resistant and therefore difficult to treat and
3 might be more virulent than other *Salmonellae*.

To cause diarrhoea or disease *Salmonella* has to survive food processing and storage. After survival of the stomach it has to adhere, invade, survive the macrophages and resist other immune system factors. In figure 1.3 the pathogenicity route of *Salmonella* is schematically presented. In the following sections of the introduction each part of the pathogenicity pathway will be described.

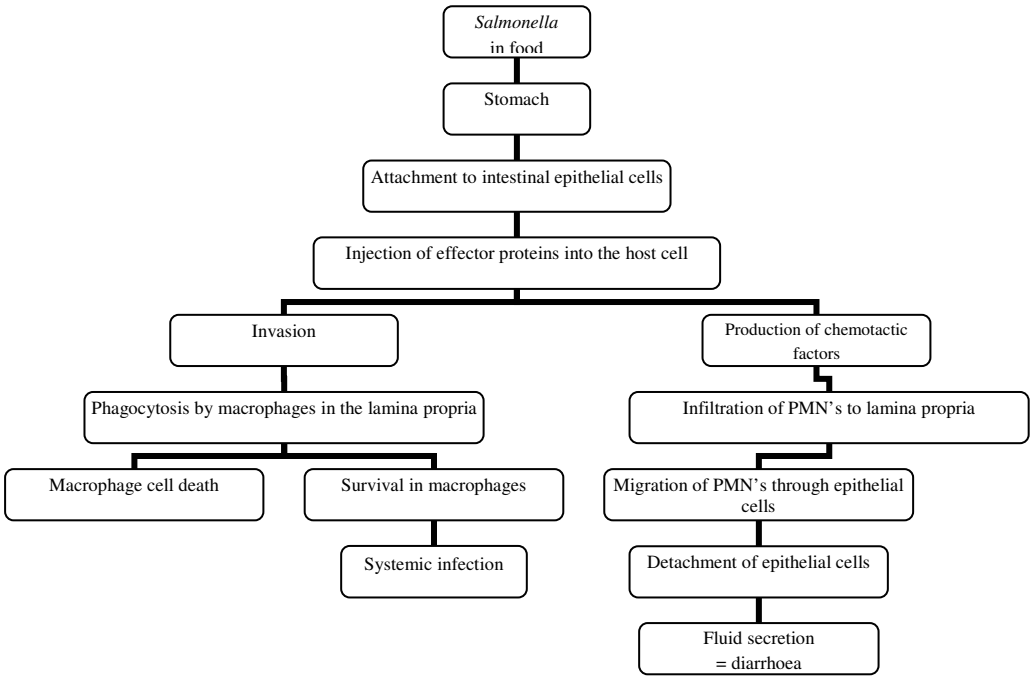


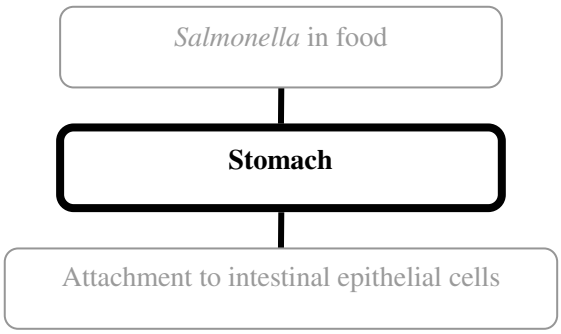
***Figure 1.3. Schematic drawing of the pathogenicity route of* Salmonella**
Pathogenesis of* Salmonella *follows two routes. In the left pathway, the bacteria invade the intestinal cells and cause systemic infectiont. In the right pathway the bacteria have an effect on the intestinal cells which leads to diarrhea. PMN= neutrophils.

FOOD

To control microbial spoilage and safety hazards in foods, traditionally heating, freezing, blanching, sterilization, curing and preservatives (like nitrate) were used. Modern preservation techniques are becoming milder in response to the consumers' demands for higher quality, more convenient foods, which are less heavily processed, less heavily

preserved, and less reliant on added preservatives. However, microorganisms like *Salmonella*, have the potential to adapt to a wide variety of these mild stresses which can lead also to cross-protection against other stresses. In addition, the use of mild stresses can lead to selection of more resistant strains that will better survive in the food product with the used preservation techniques. Due to the ability of *Salmonella* to respond to preservation stresses a higher risk may exist of *Salmonella* surviving food processing and entering the human body. These *Salmonella* are potentially both more resistant to gastric acid and more virulent and more prone to survive adverse conditions in the macrophages.

STOMACH PASSAGE



The food borne pathogen *S.* Typhimurium enters the human body via the mouth after which it reaches the stomach via the oesophagus (figure 1.4). The stomach has a fundamental role in killing pathogens present in food or water before they enter the intestinal tract. The stomach is the primary site of hydrochloride (HCl) secretion resulting in low gastric pH values, which can kill pathogens. Under fasting conditions, the pH in healthy volunteers is around 2 [14], a pH value that is lethal for most bacteria. However, gastric survival depends on a number of factors related to host, type of food and pathogen.

HOST

Reduction of the gastric acidity has been associated with an increase in the survival rates of some food-borne pathogens [15]. The acidity of the human stomach is dependent on physiological variables. The level of gastric pH can greatly differ between individuals and age groups [16]. In fasting conditions the pH of elderly is higher than that of young people. The delay of gastric transport is also associated with increasing age. Furthermore the use of different gastric acid inhibitors (e.g. Rennie®) increases the pH of the stomach. The use of gastric acid inhibitors was found to be a risk factor for infection with *Salmonella*[17].

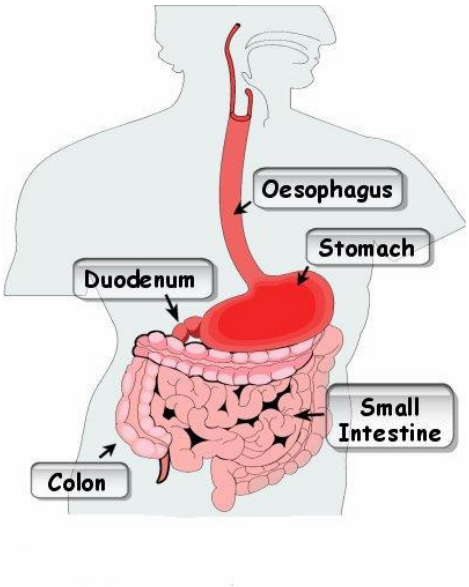


Figure 1.4. Schematic drawing of the gastrointestinal system of the human body.

FOOD

Some food components are known to protect the pathogens from the low pH of the stomach. After food intake, gastric juice was found mixed with a large number of small food particles [16;18]. The particles were probably large enough to contain one or more bacterial cells and can act as a shelter of near neutral pH value. Food-borne pathogens which are present in fatty food products like chocolate or peanut butter can escape the contact with the gastric juice by retaining in fat particles and therefore survive the stomach passage. In figure 1.5 it is shown that *Salmonella* shelters in the water fraction of olive oil surrounded and protected by fat.

In June 2001 there was a large outbreak of *S.* Typhimurium DT104 from Helva, a Turkish dessert or sweet made from sesame seeds, in Sweden, Norway, Australia, Germany and Turkey [19]. It is assumed that the high fat fraction in the food matrix protected the bacteria against the gastric acid in the human stomach as shown previously for enteric pathogens [20]. Notably, a number of *S.* Typhimurium DT104 outbreaks have previously been associated with foods containing a high fat fraction [20-24].

Ingestion of a meal can immediately raise the pH of the stomach to about 5 or 6 [25;26]. During this period, potentially disease-causing bacteria can be transported to the small intestine without being destroyed by low pH. Takumi et al.[27] showed, by modeling the gastric pH and transport, that this was indeed the case for *E. coli*. For healthy young adults 20-73% of the ingested bacteria could survive the stomach and arrived in the small intestine.

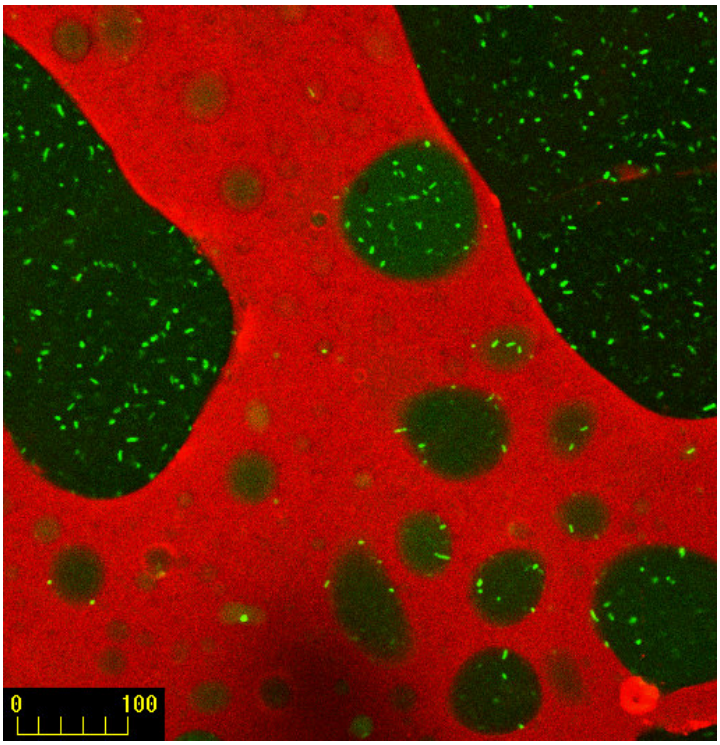


Figure 1.5.* Salmonella *(labeled green with CFDA-SE) in olive oil.* Salmonella *shelters in the water fraction of the oil. Used with permission of Rob de Jonge.

PATHOGEN

The high degree of survival during stomach passage of *E. coli* found in the study by Takumi et al. [28] was also due to the resistance of *E. coli* to low pH environments. Pathogenic bacteria, like *Escherichia coli* and *Salmonella* possess systems that assist them to adapt to and survive environmental stresses such as acidic conditions. For *S.* Typhimurium different systems have been described which protect the bacteria against low pH conditions (Fig. 1.6).

EXPONENTIAL-PHASE ATR [29]

Acid tolerance response (ATR) is the ability to survive for a prolonged period at pH 3-3.5. Exponential phase ATR is induced when *S.* Typhimurium is grown to about mid exponential growth phase in minimal media at neutral pH and involves two stages; a pre-shock which is followed by the actual low pH shock at pH 3-3.5. The pre-shock can be induced in two ways, during growth at reduced pH (e.g. 1 generation at pH 5.8) or by giving a non lethal mild acid shock (for example at pH 4.3). When exponential-phase ATR is induced during growth for 1 generation at pH 5.8 the emergency pH homeostasis is induced. By homeostasis a more neutral pH is maintained in the cytoplasm by pumping protons out of the cell or by consumption of intracellular protons. Because of the emergency pH homeostasis, at lower pH still synthesis of repair mechanisms is possible. This allows the synthesis of acid shock proteins (ASPs) at pH 3-3.5 [30]. One of the amino acid decarboxylases involved is the lysine decarboxylase [31].
When exponential-phase ATR is induced by giving a non lethal mild acid shock at pH 4.3 approximatly 50 acid shock proteins (ASPs) are formed, that are believed to prevent damage to the cell [30] during the actual low pH shock at pH 3-3.5. Three regulatory proteins control the expression of distinct sets of ASPs, the alternative sigma S factor (σ^s) (encoded by *rpoS*) [32], the ferric uptake regulator Fur (encoded by *fur*) [29;30], and the two-component sensor regulatory system PhoP/Q (encoded by *phoP* and *phoQ*) [33].

STATIONARY-PHASE ATR [34]

Studies on *S.* Typhimurium reveal two stationary-phase ATR systems, one that is acid inducible and *rpoS* independent, and one that is unresponsive to pH but *rpoS* dependent. The *rpoS*-dependent system appears to be the stationary-phase general stress resistance system, which includes resistance to low pH [32;35]. The acid inducible stationary-phase ATR is dependent on the two-component response regulator OmpR [36]. To induce the acid inducible stationary-phase ATR; bacteria are grown to stationary phase in minimal medium at pH 8, harvested and resuspended in pH 4.3 minimal medium for 2 hours and then transferred to pH 3.0 for acid challenge. Approximately 15 ASPs are formed during this ATR, of which only 5 are also involved in the exponential-phase ATR [34].

ACID RESISTANCE (AR) [37].

Other acid survival systems, that are found in *E. coli* and *Shigella flexneri* and recently also in *S.* Typhimurium[38] are called acid resistance (AR) systems.
Acid resistance is measured after a two-hour exposure to pH 2.5, a more severe acid challenge than used in the ATR. This challenge for 2 hours at pH 2.5 would represent an

average situation in the human stomach. AR measurements are done using stationary growth phase cells cultured in complex medium Luria Broth at approximately pH 5 (LB). Three complex medium-dependent AR systems have been described for *E. coli*.
AR system 1 (oxidative or glucose repressed AR) allows bacteria to survive exposure to pH 2.5 conditions in the absence of glucose. This system is highly dependent on the alternative sigma S factor (σ^s)[39].
AR system 2 (glutamate-dependent AR) provides the highest level of acid resistance, allowing bacteria to survive an extremely low pH challenge (pH 2) for several hours if glutamate is present in the challenge medium. This system is dependent on two glutamate decarboxylase isozymes encoded by the *gadA* and *gadB* genes and the putative glutamate/γ-aminobutyric acid transporter (GABA) [40].
AR system 3 (arginine-dependent AR) requires the addition of arginine in the challenge medium of pH 2.5. Arginine decarboxylase, the product of the *adiA* gene, is required for this form of acid resistance[39].
Lin et al. found that AR systems only are present in *E. coli* and *Shigella flexneri* and not in *Salmonella* [39]. A more recent study however showed that both *S.* Typhimurium DT104 and non-DT104 strains can be acid resistant and that acid resistance in *S.* Typhimurium was dependent on glucose and arginine [38].
Once induced, the acid resistance systems can persist at refrigerator temperatures for at least one month [41]. This is important when one considers that minimally processed food products are usually refrigerated for some period before consumption.

Taking into account the increase of the stomach pH after consumption of a meal, the fact that food components can protect the bacteria from contact with the low pH of the stomach and the ability of *Salmonella* to survive low pH's when adapted to mild pH, there is a high chance of *Salmonella* reaching the small intestine.

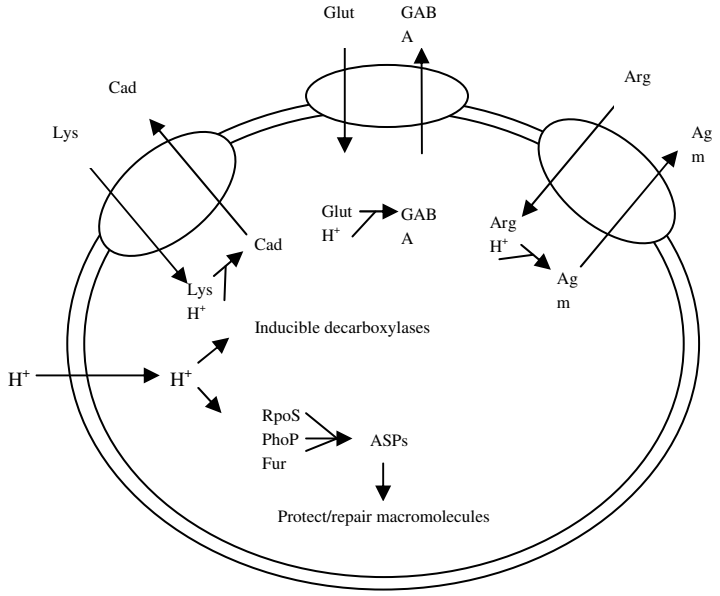
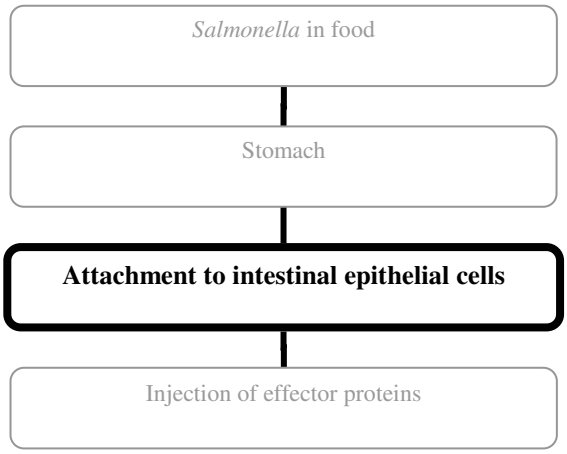


Figure 1.6. Schematic drawing of acid induced pathways and induction of decarboxylases in* Salmonella *(adapted from [42]) Lys: lysine, Cad: cadavarin, Glut: glutamate, GABA: glutamate/γ-aminobutyric acid, Arg: arginine, Agm: agmatine, ASP: Acid shock proteins.

ADHESION

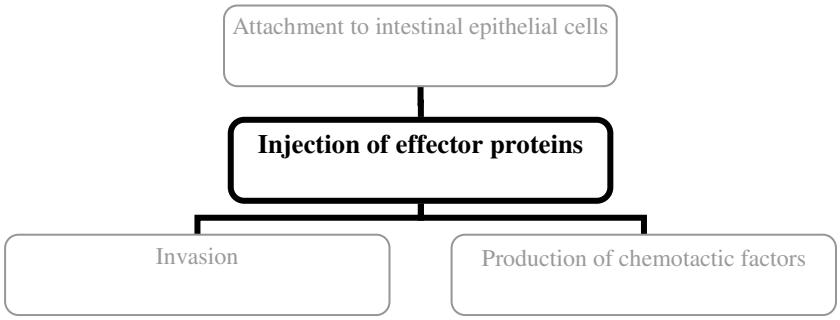


Survival of stomach passage allows *Salmonella* to enter the small intestine (fig 1.4.). To colonize the intestine, the bacteria must attach to one or more cell types and surface structures of the epithelial surface layer. For this *S.* Typhimurium contains several types of fimbriae or pili. Fimbriae were first described by Duiguid et al.[43]. and were termed type 1 fimbriae and are encoded by the *fim* operon on the *S.* Typhimurium chromosome [44-46]. These type 1 fimbriae specifically bind the extracellular matrix [47] of various eukaryotic cell types. The *S.* Typhimurium type 1 fimbriae mediate adhesion to the cervical epithelial HeLa cells *in vitro*; however, they do not appear to play any role in adhesion onto various intestinal epithelial culture cell types such as the human cell lines HEp-2, T84 and Int-407 [48]. In most *S*. Typhimurium isolates, type 1 fimbriae are the only adhesion-fimbriae expressed after cultivation in nutrient broth [49]. Another type of fimbriae, long polar fimbriae, are found to mediate adhesion to M cells, the cells of the Peyers patches, of the small intestine in a mouse model of infection [50] and are therefore important at an early step of human disease by an oral route of infection.

Genetic analysis has identified five other putative *S.* Typhimurium fimbrial operons. These are *agf* (aggregative fimbriae), *pef* (plasmid encoded fimbriae), *bcf* (bovine colonization factor), *stf* (*S.* Typhimurium fimbriae) and *saf* (*Salmonella* atypical fimbriae) [51-55]. The presence of six additional putative *S.* Typhimurium fimbrial operons, termed *stb, stc, std, sth, sti* and *stj*, was revealed by whole genome sequence analysis [56]. Thus the *S.* Typhimurium genome contains 13 (putative) fimbrial operons. Besides type 1 fimbriae, curli and plasmid encoded fimbriae, the other 10 fimbrial operons of *S.* Typhimurium appear to be poorly expressed when bacteria are grown under standard laboratory conditions [57], and no information is available about the binding specificity of the encoded adhesions. Expression of major fimbrial subunits encoded by nine fimbrial operons, BcfA, FimA, LpfA, PefA, StbA, StcA, StdA, StfA and StiA, is detectable upon recovery after infection of bovine ligated loops [57]. It could be speculated that the large number of genetic

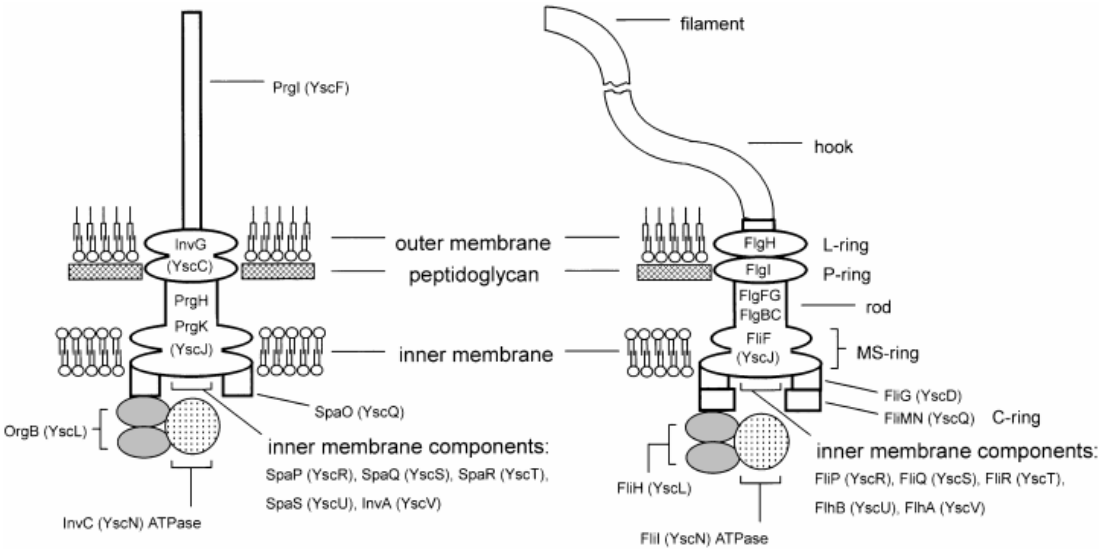
attachment factors present in *S.* Typhimurium DT104 may be the reason why *S.* Typhimurium can infect different hosts, which differ with regard to the repertoire of receptors expressed on their mucosal surfaces.

ROLE OF TYPE III SECRETION SYSTEMS IN PATHOGENESIS



After attachment of *Salmonella* to the intestinal cells different effector proteins are injected into the host cell using a type III secretion system (TTSS). TTSS are often encoded on plasmids or pathogenicity islands in host-associated Gram-negative bacteria. They transport virulence factors, termed effectors, toward or even directly into eukaryotic cells.
Salmonella is the only species described so far to contain twelve type III secretion systems, of which the two most important are encoded by two distinct gene clusters termed SPI-1 and SPI-2 (for *Salmonella* pathogenicity island 1 and 2). These two type III secretion systems appear to play different roles during pathogenesis, with the one encoded on SPI-1 being required for initial penetration of the intestinal mucosa and the one encoded on SPI-2 necessary for subsequent systemic stages of infection.
SPI-2 virulence genes are present in *S. enterica* but absent in *S. bongori*[58;59]. Both clusters have presumably been acquired independently[60], with SPI-2 being the more recently acquired. *Salmonella* may have initially evolved to a pathogen capable of localized gastrointestinal infections (SPI-1 function) and later on extended the pathogenic potential towards systemic infections (SPI-2 function). SPI-1 is located at centisome 63 of the *Salmonella* chromosome[61;62]. The 40 kb SPI-1 region encodes at least 33 proteins; which consist of regulatory proteins, secreted effector proteins and their chaperones. The fact that different *Salmonellae* encode different effectors proteins, suggests that TTSS may be of importance in the host specificity of different *Salmonella*. Electron micrographs of *S.* Typhimurium reveal that the SPI-1 TTSS resembles the multi-ringed flagellar basal body

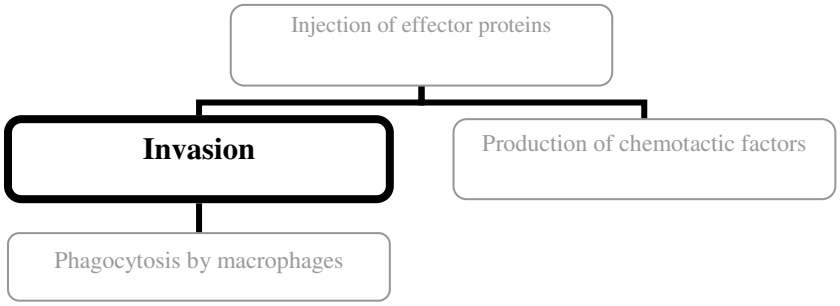
with a hollow needle-like barrel projecting out of its center [63] (figure 1.7). TTSS-1 effectors probably pass directly from the bacterial cytosol into the host cell cytosol via this channel. The expression of genes encoding the TTSS-1 apparatus and most of its effectors requires HilA, a transcription factor encoded on SPI-1[64;65]. Osmolarity, oxygen and pH coordinately affect the transcription of *hilA* and changes in the level of HilA mediate the regulation of TTSS-1 by the same environmental conditions[66;67]. The SPI-1 TTSS of *S.* Typhimurium delivers at least 13 effector proteins through the host cell plasma membrane. These effector proteins are important for the invasion of *Salmonella* into the intestinal cells and for inducing an immune response in the host.



SPI-1 TTSS Flagellar basal body

Figure 1.7. Schematic figure of the resemblance of* Salmonella *SPI TTSS and the flagellar basal body (copied from http://mcb.berkeley.edu/courses/mcb103/02BacterialCellSFII.htm).

INVASION OF *SALMONELLA* INTO INTESTINAL CELLS



Bacteria that survive the stomach barrier can subsequently adhere and colonize the intestine and invade the intestinal cells. M cells have often been suggested as the primary invasion site for *Salmonella spp* [68;69]. It is clear however that *Salmonella* can also breach the intestinal barrier through the absorptive columnar epithelial cells (enterocytes) of the small intestine[70], and it has been argued that perhaps enterocytes may indeed constitute the main port of *Salmonella* entry since they vastly outnumber M cells[71].
Different environmental conditions in different parts of the intestine influence the expression of invasion genes (Table 1.1). The effects of these environmental conditions cause the invasion of *S.* Typhimurium to take place mainly in the last part of the small intestine, the ileum. Invasion of intestinal cells results in the extrusion of infected epithelial cells into the intestinal lumen with consequent villus blunting and loss of absorptive surfaces. The invasion pathway is shown in figure 1.8. Invasion is necessary to cause a systemic infection.

Table 1.1 Effect of environmental conditions in the intestine on invasion

Condition	***localisation***	***effect***	***reference***
Low oxygen	**whole intestine**; the lumen is anaerobic and the brush border of the epithelial cells is microaerophilic	SPI-1 genes are maximally expressed through HilA	[72;73;74]
High osmolarity	**small intestine;** greater than 300 mOsm	stimulate invasion	[72]
High bile concentration	**duodenum;** Bile is mainly produced in the proximal small intestine	Bile represses SPI-1 genes	
Acetate concentrations	**ileum;**	induces invasion and the expression of SPI-1	[75;76]
Propionate and butyrate concentrations	**coecum and colon;** high concentrations	repress SPI-1 gene expression	[76]

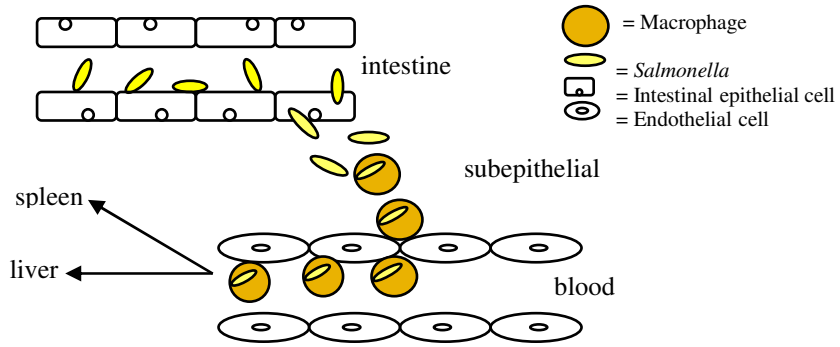
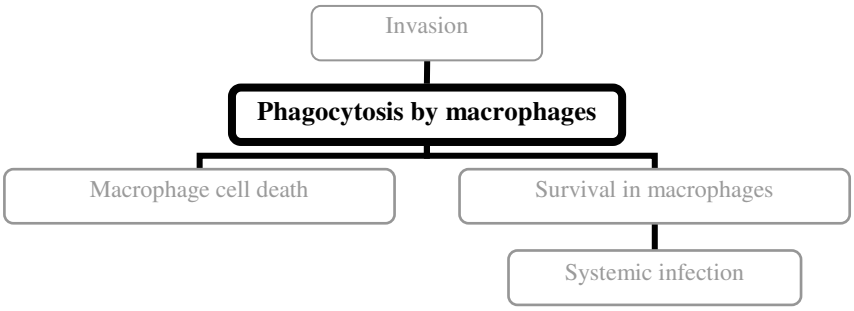


Figure 1.8. Schematic drawing of the invasion and translocation of* Salmonella *across the intestinal wall and transfer to secondary organs, like liver and spleen.

MACROPHAGES



After *Salmonella* has penetrated the epithelial cells of the small intestine it enters the subepithelial space. In this subepithelial space, cells from the human immune system are present. These are mainly white blood cells. Macrophages are white blood cells that can also remain in the tissue and are important in clearing extracellular bacteria and parasites. The macrophage's NADPH oxidase is a multicomponent enzyme that produces superoxide by the reduction of molecular oxygen. Many microbes are sensitive to superoxide and its derivates, including hydrogen peroxide and hydroxyl radical, collectively known as reactive oxygen species (ROS). In macrophages with active inducible nitric oxide synthase (iNOS), superoxide can react with nitrogen oxide (NO) to generate peroxynitrite and other reactive nitrogen species (RNS).

In the subepithelial space *Salmonella* can be phagocytosed by macrophages. Although macrophages are designed to kill bacteria, *Salmonella* can survive and replicate in the macrophage [77]. Residing in macrophages protect *Salmonella* from other cells of the immune system. Moreover macrophages also transport the *Salmonella* through the bloodstream to other organs (e.g. liver and spleen), so *Salmonella* can cause a systemic infection throughout the host. [78-84].

Salmonella possesses several enzymes which can defend it from oxidative stress caused by ROS e.g. periplasmic superoxide dismutases, catalase and glucose 6-phophate dehydrogenase [85-87]. Despite the range of enzymes utilized by *Salmonella* to reduce or repair oxidative damage, virulent *S.* Typhimurium are still highly susceptible to phagocyte killing in the first few hours after phagocytosis by macrophages [88]. Therefore *Salmonella* uses another strategy that protects it from killing by the macrophages. After phagocytosis

by the macrophages *Salmonella* remains in the phagosome. Normally this phagosome fuses with a lysosome, leading to the killing of bacteria by the NADPH oxidase. However, upon phagocytosis by macrophages, the phagosome containing *S.* Typhimurium remains segregated from the lysosome [89-91].

The vacuole in which the *Salmonella* remains is called the *Salmonella* containing vacuole (SCV). SPI-2 function is required to inhibit maturation of *Salmonella* containing phagosomes to phagolysosomes. It has been reported that SPI-2 effector SpiC, is involved in the segregation of SCVs from the late endocytic pathway [89]. SifA, another effector protein of SPI-2, is required to maintain the vacuole around *Salmonella* [92]. Although the SCV is separated from the lysosome, it rapidly acquires the lysosomal membrane glycoproteins, LAMP-1 and LAMP-2, and acidifies to pH 4.0-5.0 within an hour of uptake [90;93]. Vacuolar acidification is required for *Salmonella* replication in the SCV [94;95]. Under laboratory conditions growth in minimal media with low Mg^{2+} or Ca^{2+}, as well as starvation, are environmental conditions that can induce SPI-2 gene expression. These are: nutritional limitations that might also be sensed inside the phagosome of infected cells [94].

SPI-2 mutants show wild-type phenotypes in the interaction with the intestinal epithelium and cause diarrhea in different animal models [96;97], but SPI-2 mutants are defective in intracellular parasitism, i.e. survival and growth in host cells, like macrophages [89;98-100]. SPI-2 gene expression is modulated but not directly controlled by the two-component system PhoPQ [101]. Another two-component system, OmpR-EnvZ may act as a sensor for low osmolarity and directly regulate the expression of *ssrAB*, two genes that are absolute required for SPI-2 gene expression [102]. At present 13 effector proteins translocated by the SPI-2 encoded TTSS have been identified. For most effector proteins the function is not yet determined, others appear to be important for forming microtubuli between different SCVs[103]
Also SPI-1 encoded type III secretion system has been shown to affect the interaction of *Salmonella spp.* with cultured murine macrophages. However, SPI-1 does not affect the ability to persist and replicate in macrophages, but mediates a cytotoxic effect [104].

The outcome of *Salmonella* inactivation with macrophages depends on the relative contribution of both SPI-1 and SPI-2 TTSS, in conjunction with the stimulation of innate immunity outputs conserved determinants collectively known as ‘pathogen-associated molecular patterns’ (PAMPs). These interactions result in a breakdown of the balance between survival and pro-apoptotic cellular pathways, which eventually leads to macrophage cell death. (figure 1.9).

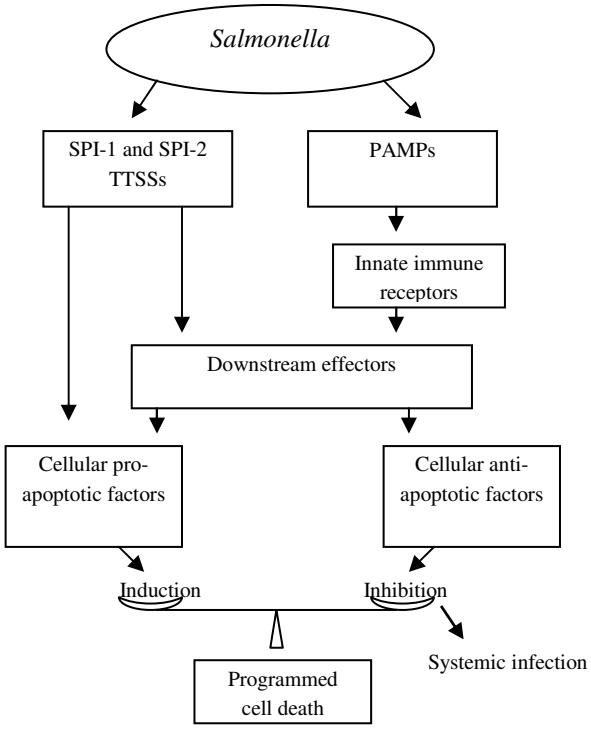
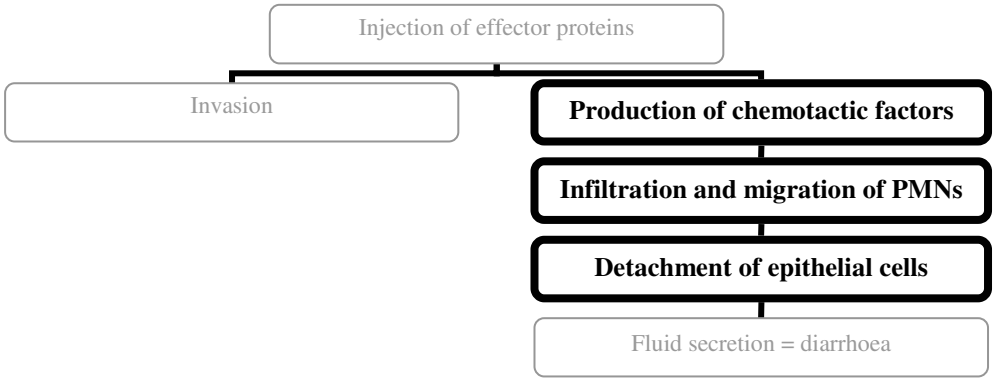


Figure 1.9. Schematic drawing of the outcome of* Salmonella *activities in macrophages. PAMPS: pathogen-associated molecular patterns.

IMMUNE RESPONSE



In addition to invasion of epithelial cells, the SPI-1 system appears to function in programmed cell death of infected cultured macrophages [104;105]. Next to these functions the SPI-1 system also functions in the recruitment of other white blood cells called polymorphonuclear neutrophils (PMNs) to the small intestine [106] causing inflammatory diarrhoea in certain hosts [107].

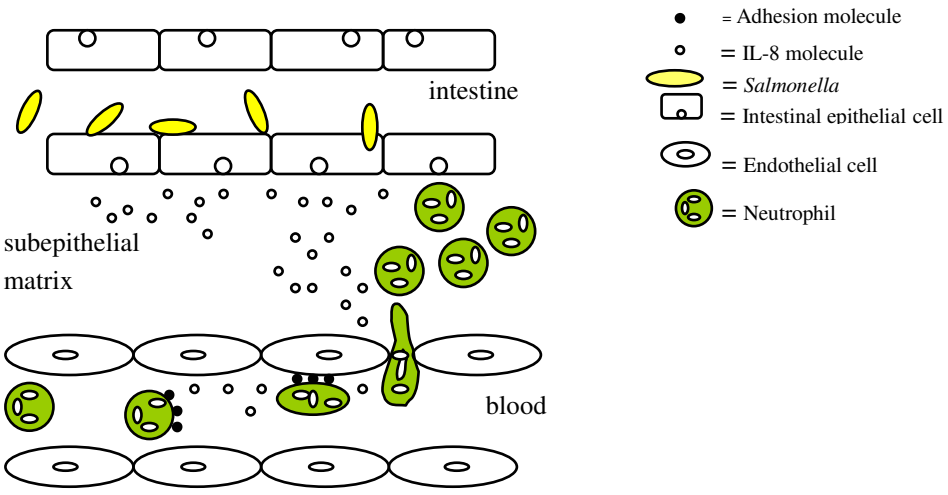


***Figure 1.10. Schematic drawing of the immune response of intestinal cells to* Salmonella.**

In vitro studies have shown that *Salmonella* interaction with epithelial cells results in a proinflammatory response characterized by the release of several cytokines and chemokines [108], with interleukine-8 (IL-8) being the best studied.
Infection of epithelial cells with *Salmonella* causes via Toll like receptors (TLR), the release of IL-8 on the basolateral side, setting up a gradient that promotes PMN migration through the matrix (figure 1.10).

In addition, infection of the polarized epithelial cells causes the release of a new PMN chemoattractant, pathogen-elicited epithelial chemoattractant (PEEC), on the apical surface, which stimulates transmigration of the PMNs through the epithelium [109]. Thus infection of the epithelium by *Salmonella* causes basolateral release of IL-8, resulting in migration of PMNs into the subepithelal space and apical release of PEEC, in turn resulting in intraepithelial migration of the PMNs with subsequent release into the lumen.

Studies performed *in vitro* and in a bovine model system suggest that the SPI-1 secretion apparatus and secreted effectors SopB and SopE promote chemokine production and recruitment of PMNs across the intestinal epithelium[110]. Neutrophils are proposed to play a very important role in the pathogenesis of *Salmonella*-induced diarrhoea, since experimental depletion of the PMN pool by administration of nitrogen mustard to rabbits results in a significant decrease in intestinal fluid secretion [111] and administration of an anti-inflammatory agent (indomethacin) completely abolishes fluid secretion in rabbit intestinal loops inoculated with *S.* Typhimurium [112]. More recently it was found that the inflammatory response, characterized by neutrophil infiltration, precedes intestinal fluid secretion in calves [113].
Whether an inflammatory PMN response is beneficial to the bacteria, the host, or possibly both, remains to be determined. It might benefit the bacteria, especially if *S.* Typhimurium can reside inside PMNs. An inflammatory PMN response might also benefit the bacteria if recruitment of inflammatory cells promotes bacterial growth and spread throughout the intestinal mucosa by compromising the intestinal barrier. By increasing bacterial shedding PMN recruitment could increase bacterial spread throughout a host population. In contrast, increased intestinal fluid secretion can also result in wash out of bacteria through the intestinal lumen, which is beneficial for the host, although the host becomes ill and suffer from diarrhoea.

INFECTIOUS MODELS

The virulence of *Salmonella* and other pathogens and the effect of different strains and growth conditions can be determined in several systems, like cell lines, animal or in human volunteers. In the following sections an overview will be presented.

CELL LINES

Several human intestinal cell lines are available to study interactions of *Salmonella* with epithelial cells. The human adenocarcinoma cell lines, Caco-2, HT-29 and T84, have been shown to form polarized monolayers and well-defined brush borders, mimicking the human intestinal epithelium. The Caco-2 cell line is known to spontaneously differentiate to form such a polarized cell layer [114]. Although the cells are isolated from an carcinogenic adult human colon and they express several markers characteristic of normal small intestinal villus cells [115]. The Caco-2 cell line has been shown to be a good *in vitro* system for the analysis of *Salmonella* virulence characteristics. One study [116] showed that data obtained with the Caco-2 cell line correlate well with results obtained with animal infection models for *S.* Typhimurium and *Salmonella* Cholearaesuis [70]. Also Caco-2 data for *S.* Typhimurium [117] have been shown to correlate with the rabbit ileal invasion assay. The latter approach is expected to provide solid data on virulence, since it is based on an *in situ* assay.
In vitro virulence of *S.* Typhimurium DT104 has previously been determined using Hep-2 cells [118;119] and undifferentiated intestinal cells (INT-407) [120] [121] but never in fully differentiated intestinal cells such as Caco-2 cell lines. It is expected that the latter approach mimics the *in vivo* infection pathway more closely because of the similarity of Caco-2 cells with the epithelial cells of the ileum i.e. the site of *Salmonella* invasion. Next to human intestinal cell lines also intestinal cells derived from different animals can be used.
The rat ileal crypt-derived non-transformed cell line IEC-18, was originally isolated to investigate cytotoxic effects of chemical carcinogens. In the early nineties filter-grown IEC-18 cells were used as a model to study small intestinal epithelial permeability. It was found that filter-grown IEC-18 developed permeability comparable to that of the small intestine [122].
The advantage of using cell line models is that it is a simple model. Most variables are controllable and can easily be manipulated. The fact that this model is simple is also its disadvantage, since it is a simplification of reality and therefore data are missing on the interaction between different cells, organs and the immune system. Cell line models are therefore useful in studying the initial steps of infection, like adhesion, invasion and the induction of IL-8 production.

ANIMAL MODELS

The advantage of using animal models compared to cell lines is the fact that a whole organism can be studied, including the immune response. However it is important to select an appropriate animal model, which assumes that the pathogen causes disease by the same mechanism of pathogenicity in both man and animal. The animal's physiological and immune responses and the relationships between infectivity, morbidity and mortality have to be similar to that of man. *S.* Typhimurium infections in mice are often used to study the effects of *S.* Typhi in man, therefore mice do not seem to be the perfect animal to use to study *S.* Typhimurium infection in man, although recently Stecher *et al.*[123]described a new mouse model with predominantly intestinal disease.

Rat provide a useful model for some aspects of salmonellosis in humans, as intestinal transport and electrolyte transport patterns, important factors in salmonella infections, are similar in rats and in humans [124]. Within 2 hours after oral exposure of rats to *Salmonella*, the bacteria can be detected in the distal ileum and coecum. Invasion occurs via the M-cells in Peyers patches and the *Salmonella* can be detected in the mesenteric lymph nodes within 8 hours after infection [125]. Rats develop clinical disease only at doses higher than 10^8 colony-forming units (cfu) when administered orally [126] [127]. The differences in clinical responses imply that the rodent is particularly useful for investigating the initial stages of the pathological pathway [28].

HUMAN VOLUNTEER STUDIES

The primary source of dose-response data has been human volunteer feeding studies. Such trials provide the most direct measure of human response to pathogens, which is its big advantage. Another advantage is that these studies are obtained under well-controlled conditions and can therefore be subjected to detailed mathematical analyses[128]. Also the volunteers can be closely monitored for signs of infection and symptoms of illness. However, these data do have limitations. Volunteers for these studies have been almost exclusively healthy adult males. Information on the susceptibility of higher risk subpopulations or potential gender effects is generally not available. Furthermore, volunteer studies are limited to food borne diseases that are not considered life-threatening for the test subjects. Also it is usually not possible to evaluate dose levels that are related to the pathogen levels associated with human exposures via food, since a high amount of volunteers are needed to obtain statistical significant results. Nine studies have been published of experimentally induced salmonellosis, conductucted between 1936 and 1970 using a variety of serotypes and strains[129-137]. Although the list of human feeding trials for *Salmonella* in humans is more extensive than may exist for other bacterial pathogens, some

of these studies are unsuitable for further analysis on the pathogenicity of *Salmonella* in humans. Three of these studies used only one high dose of *Salmonella* with a 100% attack rate[129;130;135]. Some other studies are conducted using *S.* Typhi[134;136;137], of which the pathogenicity is not comparable with that of non-typhoid *Salmonella* like *S.* Typhimurium. The most extensive human feeding trials of non-typhoid *Salmonella* were conducted in the late 1940s to early 1950s by McCullough and Eisele[131-133;138]. Six different *Salmonella* serotypes were used, with up to 3 or 4 different strains of some of the serotypes. The subjects used in the feeding trials were healthy males from a penal institution. The dose was administered in a glass of eggnog shortly following a noon meal. The feeding trial data have been reviewed and critiqued by for example Blaser and Newman[139]. Although concerns have been raised as to the experimental design of the human feeding trials, these data provide a basis for dose-response relationship of *Salmonella.*

PARALLELOGRAM APPROACH

It is difficult to extrapolate the *in vitro* cell-line data and *in vivo* data obtained by animal studies to the human situation. To extrapolate experimental data to man the parallelogram approach was proposed in the late 1970's by Dr. Frits Sobels[140]. It was originally designed for extrapolation of chemical mutagenesis data. In this approach (see fig. 1.11) data derived from different *in vitro* cell systems can be compared with each other (A) and data from experiments with animal cells can be compared with *in vivo* animal data (B). Extrapolation can now follow two routes: from *in vivo* animal data to man (C) and from *in vitro* human data to man (D).

The first step (A) in this approach is to compare different *in vitro* systems with each other. This has to be a human cell line from a certain cell type and an animal cell line from the same cell type. Different effects on these cell lines can be studied like; cell death or the induction of a certain response by this cell line. Next a (mathematical) relation has to be made between the different cell lines. The second step (B) in the parallelogram approach is to compare *in vitro* animal data with *in vivo* animal data. For this step a parameter *in vitro* has to be chosen in the cell line which corresponds with a parameter *in vivo* in the animal model, therefore the underlying mechanism of the effect that will be studied has to be known. The cell line used must be of the same origin as the animal organ to be studied. For example, if the effect of cigarette smoke on the development of lung cancer will be studied in a mouse model, preferable a cell line from lung cells of a mouse should be used. After measuring the parameter in the cell line which is linked to the parameter measured in the animal a (mathematical) relation has to be made between these two parameters.

The last step is to make a translation to human. Both routes of the parallelogram approach can now be used to predict the effect in humans. The relations between *in vitro* human and animal data (A) and between animal *in vitro* and *in vivo* data (B) are known. The data obtained with *in vivo* animal experiments and relation A can be used to predict the human risk (C). Additionally the data obtained with human cell lines *in vitro* and relation B can also be used to predict the human risk (D). Although, this approach is used often for the prediction of chemical risks, it has never been used for predicting the microbiological risks in humans.

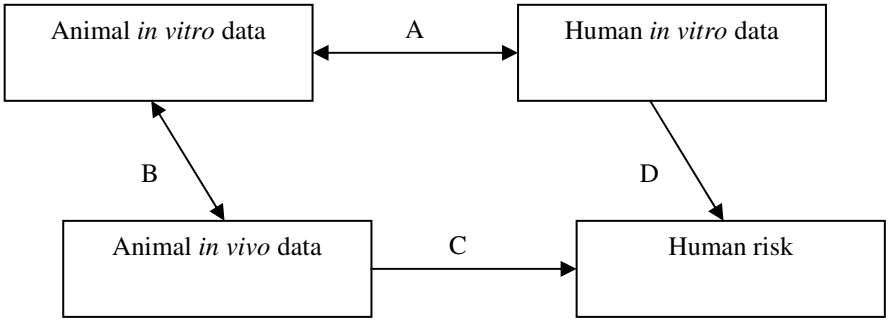


Figure 1.11. Parallelogram approach.

OULINE OF THE THESIS

The research in this thesis was initiated because the incidence of multi-antibiotic resistant *Salmonella* Typhimurium DT104 in 2001 had increased in many countries including the Netherlands. There was evidence that the clinical symptoms of this *Salmonella* were more severe than other *S.* Typhimurium or *S.* Enteritidis infections. However it is not known at which level of the pathogenicity pathway this increased virulence is found and what virulence factors are involved. Therefore different steps in the pathogenetic pathway of *Salmonella* are studied. The effects of different growth conditions, representing food relevant conditions, are studied in the different steps of the pathogenic pathway.

Since *S.* Typhimurium is a food borne pathogen it enters the human body via the acidic stomach. In **Chapter 2** the level of acid resistance was determined for 37 *S.* Typhimurium DT104 strains to determine the capability of surviving the low pH of the stomach. Strains isolated from different food products as well as strains isolated from humans have been tested for their level of acid resistance, to investigate if differences in acid resistance can be linked to their isolation sources.

When *Salmonella* has passed the stomach it enters the intestines. In the intestines the bacteria have to adhere and invade the intestinal cells to cause a systemic infection. In **Chapter 3** the adhesive and invasive capabilities of 5 *S.* Typhimurium DT104 strains initially characterized in Chapter 2 are examined *in vitro* in Caco-2 cells, a human intestinal cell line. Also the ability to cause an immune response by the host cells is determined; this is done by measuring the IL-8 production by Caco-2 cells after exposure to *Salmonella*.

To study the effects of a *S.* Typhimurium infection in an animal model, rats are infected with *S.* Typhimurium DT104. In **Chapter 4** the results of these experiments are described. In this chapter the dose-response relationship is determined and a study on the induction of the immune response is described. In addition the effect of growth pH and growth phase on *Salmonella* virulence is studied in a rat model.

Next to these *in vivo* studies, also *in vitro* studies are performed using rat and human cell lines, in order to assess the impact of strain diversity, growth phase and growth pH on *Salmonella* pathogenicity. In **Chapter 5** the relationship between the rat and human cell line is described and also the relationship between *in vitro* results in the rat cell line and *in vivo* results in the rat is described. By using a parallelogram approach the relation between *in vitro* results obtained in the human Caco-2 cell line and the probability of infection in human is estimated. **Chapter 6** includes a general discussion.

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CHAPTER 2

Acid resistance variability among isolates of *Salmonella enterica* serovar Typhimurium DT104

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Published in Journal of Applied Microbiology 2005: 99; 859-866

ABSTRACT

Aims: Acid resistance could be an indicator of virulence since acid resistant strains are able to better survive the human stomach passage and in macrophages. We studied the acid resistance of several *S.* Typhimurium DT104 strains isolated from food and patients and identified cellular parameters contributing to the enhanced acid resistance of these isolates.
Methods and results: Acid resistance was tested in 37 *Salmonella enterica* Typhimurium serovar DT104 (*S.* Typhimurium DT104) strains, isolated from both human and non-human sources, and in 2 reference strains. Acid adaptation at pH 5 followed by exposure for 2 hours at pH 2.5 revealed strong variation of acid survival. After two hours at pH 2.5 six strains of *S.* Typhimurium DT104 were considered high acid resistant since they displayed a level of survival greater than 10%, fourteen strains were considered intermediate acid resistant (level of survival was smaller than 10% and greater than 0.01%) and nineteen strains were considered low acid resistant (level of survival smaller than 0.01%). Six strains were selected for further studies and proteomics revealed a relatively low amount of phase 1 and phase 2 flagellins and a relatively high amount of the beta component of the H^+/ATPase in the acid resistant strains. Two strains were slightly more heat resistant possibly as the result of increased levels of DnaK or GroEL.
Conclusions: A significant difference could be detected between human and food isolates regarding to their acid resistance; all high acid resistant strains were human isolates.
Significance and Impact of the Study: *S.* Typhimurium DT104 is known for two decades and has a great impact on human health causing serious food borne diseases. Our results suggest the existence of a positive correlation between acid resistance and pathogenicity in *S.* Typhimurium DT104 since all high acid resistant strains were isolated from humans.

INTRODUCTION

During the last decades new emerging food borne pathogens have been identified. There are several reasons for the emergence of food borne pathogens: (I) Globalization of the food supply; raw materials and food from all over the world are imported and consequently also the pathogens in these foods. (II) International travelers, refugees, and immigrants also bring different pathogens with them, including food borne pathogens. (III) Change in the human population, like ageing, malnutrition, HIV, and other underlying diseases that favor the probability of an infection with pathogens, including food borne pathogens. (IV) Consumers demands; consumers want easy to handle and healthy food, which means that foods are less heavily processed to minimize the loss of vitamins and taste, with the risk of survival of food borne pathogens in these foods [1]. And last but not least, (V) the evolution of new strains. New properties including antibiotic and stress resistance and toxin production may be acquired via (horizontal) gene transfer and may be located on plasmids [2].

Salmonella enterica serotype Typhimurium definitive phage type DT104 (*S.* Typhimurium DT104) was first reported in the UK in 1984 [3;4]. The epidemic strain has an antibiotic resistance pattern characterized by resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (ACSSuT) that is located on the *Salmonella* genomic island 1 (SG-1) [5]. During the last decade there is a large increase of *S*. Typhimurium DT104 in the Netherlands [6;7]. In the period 1990 to 2001 the percentage of all human *Salmonella* isolates typed DT104 in the Netherlands rose from 0.66 to 18% in 2001 probably promoted by transportation of manure between farms [7]. Several reports have indicated that these emerging *S.* Typhimurium DT104 are more virulent in humans than other *Salmonella's*. Wall *et al*. [3] found a higher mortality and morbidity in patients infected with *S.* Typhimurium DT104. *S.* Typhimurium DT104 strains were found to be more acid resistant [8] than other *Salmonella's.* Since the stomach with its acidic pH is recognized as the first line of defense against food borne pathogens, acid resistance of pathogens may contribute to their virulence.

For this study we collected 37 *S.* Typhimurium DT104 strains and tested them for acid resistance and investigated whether human isolates are more acid resistant than non-human isolates. Therefore, the sequence of *rpoS* (the master regulator of stress response in *Salmonella*), heat resistance, and 2D protein profiles of high acid resistant and low acid resistant strains were determined to investigate whether these strains differ not only in acid resistance but also in other stress response determinants.

MATERIALS AND METHODS

BACTERIAL STRAINS AND CULTURE CONDITIONS

37 *Salmonella enterica* serovar Typhimurium DT104 isolates were collected from both human and non-human sources. Two *Salmonella* reference strains were included, BAA-188 a human DT104 isolate from the American Type Culture Collection (ATCC) and LT-2, a *Salmonella* Typhimurium PT2 strain that was recently sequenced [9] (see Table 2.1).
All strains were stored at -70°C in Brain Heart Infusion broth (BHI) (Brunswich Chemie, Amsterdam, the Netherlands) containing 50% glycerol. Strains were allowed to recover from –70°C for 8 hours at 37°C in BHI without shaking after which the bacteria were used in the different experiments. Growth media consisted of LBG (10 g tryptone, 10 g NaCl, 5 g yeast extract and 4 g glucose per liter) buffered with 100 mM 2-morpholine-ethanesulfanic acid monohydrate and set at pH 5.0. LBG was inoculated with 1% BHI culture and cells were grown for 18 hours without shaking at 37°C to obtain stationary phase cultures.

ACID RESISTANCE

Studies were conducted in closed 30 ml bottles containing a volume of 25 ml. Bacterial cultures were grown overnight for 18 hours without shaking in buffered LBG pH 5 at 37°C. The overnight cultures yielded approximately 10^8 colony forming units (CFU) per ml. Cultures were acidified to pH 2.5 using 6 M HCl and subsequently 5 ml was added to 25 ml fresh buffered LBG pH 2.5, and incubated at 37°C. Samples were taken before acidification (t=0) and after 1 and 2 hours at pH 2.5 and subsequently incubated for 24 hours at 37°C after dilution in peptone physiological salt solution (ppss) containing 9 g NaCl and 1 g peptone per liter and plating on tryptone soy agar (TSA). Survival was calculated as percentage survival at t = x: AR = $(CFU_{t=x}/CFU_{t=0}) \times 100\%$. Strains of which the level of survival was greater than 10% after 2 hours at pH 2.5 were considered high acid resistant, whereas strains of which the level of survival was smaller than 0.01% were considered low acid resistant. All other strains were considered intermediate acid resistant.

HEAT RESISTANCE

Bacterial cultures were grown overnight for 18 hours without shaking in buffered LBG pH 5 at 37°C. Cultures were transferred (10%) in prewarmed LBG pH 5.0 and incubated at 55°C in 10 ml tubes. Samples were taken directly and after 1 and 2 hours at 55°C and subsequently diluted in ppss and plated on TSA and incubated for 24 at 37°C. Survival was calculated as percentage survival at t = x: HR = $(CFU_{t=x}/CFU_{t=0}) \times 100\%$.

TOTAL CELLULAR PROTEIN EXTRACTION

Cells were harvested by centrifuging 10-ml cultures for 10 minutes at 3.000 × g and the pellet was resuspended in water containing a protease inhibitor (5 mM pefabloc). Subsequently, cells were disrupted by bead beating (three times for 1 min) with zirconium beads (0.1-mm diameter; Biospec Products, Bartlesville, USA) in a MSK cell homogenizer (B. Braun Biotech International, Melsungen, Germany). In between the treatments the cell extract was cooled on ice. The protein concentration in cell extracts was determined by using the bicinchoninic acid assay (Sigma Chemical Co., St. Louis, USA).

PROTEIN ANALYSIS BY 2D ELECTROPHORESIS

Protein analysis was performed with a Multiphor 2D-EF system (Pharmacia Biotech, Uppsala, Sweden) as described by the manufacturer. Essentially, prior to loading the samples on the IEF gel 40 µl of protein (40 µg) was treated with 40 µl lysis solution [9 M urea, 2% 2-mercaptoethanol, 2% IPG buffer 4-7 (Pharmacia Biotech), 2% Triton X-100] at room temperature for 2 min, after which 80 µl sample solution (8 M urea, 2% 2-mercaptoethanol, 2% IPG buffer 4-7, 0·5% Triton X-100, a few grains of bromophenol blue) was added. At the acidic end of the first-dimension IEF gels with linear pH ranges from 4 to 7 (Immobiline Dry strips, Pharmacia Biotech) the total volume (160 µl) was loaded. Subsequently, the proteins were separated on precast gradient sodium dodecyl sulfate-polyacrylamide gels (12 to 14%) (Pharmacia Biotech). The gels were silver-stained as described by Blum *et al.* [10]. The experiments were performed in duplicate or triplicate, and representative gels are shown. The gels were analyzed, integrated, and normalized by using PD-Quest software (Bio-Rad).

DETERMINATION OF AMINO ACID IDENTITIES USING MALDI-TOF MS

Protein samples (750 µg) were separated on the 2D-EF gels under conditions identical to those used for the running of analytical gels. Gels were stained with Coomassie Brilliant Blue and subsequently Maldi-TOF MS analysis of manually excised spots was carried out at the Maastricht Proteomics Centre (Department of Human Biology, University of Maastricht, Maastricht, The Netherlands) by in-gel trypsin digestion in a Mass Prep-station and subsequent analysis with a Maldi-TOF LR Mass spectrometer (Micromass, Almere, The Netherlands). Peptide mass fingerprints were analyzed using Mascot MS/MS search engine (Matrix Science Ltd., London, United Kingdom).

SEQUENCING OF THE RPOS GENE

DNA sequences of both strands of the *rpoS* gene were determined by primerwalking. Therefore, the coding region of *rpoS* was amplified using Pwo DNA polymerase (Roche

Diagnostics, Almere, the Netherlands) on a MJ PTC-200 thermalcycler (Biozym, Landgraaf, the Netherlands). Nucleotide sequencing reactions were performed with purified PCR products using AmpliTaq FS DNA polymerase Fluorescent dye terminator reactions (Perkin-Elmer) as recommended by the supplier. Sequencing products were detected using an Applied Biosystems 373A stretch automated DNA sequencer. Nucleotide sequence analysis was performed with the Lasergene analysis package (DNASTAR).

Table 2.1 Low pH survival and acid resistance of* S. *Typhimurium DT104 strains from humans and foods.

Strain	Isolation source	Source	Survival after 1 hour (%)	Survival after 2 hours (%)	Acid resistance
***S.*Typhimurium DT104**					
325	tahini[1]	Folkehelsa[2]	2.3	0.0020	low
327	tahini	Folkehelsa	0.19	0.00014	low
392	human, stool	Folkehelsa	0.13	0.0029	low
394	helva[1]	Folkehelsa	2.3	0.0026	low
401	helva	Folkehelsa	1.2	0.0027	low
402	human, stool	Folkehelsa	25	0.015	intermediate
636	human, stool	Folkehelsa	1.7	0.0026	low
637	sera	Folkehelsa	7.3	0.00053	low
645	sera	Folkehelsa	0.68	0.0016	low
910	human,blood and stool	Folkehelsa	0.69	0.00052	low
911	human, stool	Folkehelsa	0.25	0.00027	low
951	human, stool	Folkehelsa	0.17	0.0028	low
953	human, stool	Folkehelsa	1.2	0.0016	low
1242	helva	Folkehelsa	0.71	0.0022	low
1243	helva	Folkehelsa	1.9	0.0020	low
2929	human	RIVM[3]	78	2.7	intermediate
2945	human	RIVM	73	36	high
2946	human	RIVM	74	35	high
2975	human	RIVM	35	33	high
3001	human	RIVM	0.34	0.0072	low
3025	human	RIVM	0.036	0.00010	low
3086	human	RIVM	39	34	high
3087	human	RIVM	0.53	0.023	intermediate
3088	human	RIVM	42	33	high
3089	human	RIVM	33	0.049	intermediate
3123	human	RIVM	36	33	high
3145	human	RIVM	0.29	0.0079	low
3146	human	RIVM	35	0.059	intermediate

Table 2.1 (continued)

Strain	Isolation source	Source	Survival after 1 hour (%)	Survival after 2 hours (%)	Acid resistance
***S.*Typhimurium DT104**					
3253	human	RIVM	55	2.3	intermediate
3260	human	RIVM	55	0.29	intermediate
3272	human	RIVM	51	2.7	intermediate
3279	human	RIVM	48	0.11	intermediate
3292	human	RIVM	54	2.1	intermediate
3384	meat	RIVM	0.034	0.00047	low
3467	human	RIVM	54	2.2	intermediate
3633	cocoa	RIVM	27	0.88	intermediate
3635	nuts/seeds	RIVM	21	0.37	intermediate
BAA-188	human	ATCC[4]	5.98	0.033	intermediate
***S.* Typhimurium**					
LT-2	livestock	ATCC 700720	0.037	0.00014	low

Acid survival of stationary phase S. *Typhimurium DT104 strains was determined after 1 and 2 hours at pH 2.5. Acid resistance was calculated as percentage survival at t = x AR = ($cfu_{t=x}/cfu_{t=0}$) ×100%.*
[1] Helva, also known as halva, and tahini are Middle Eastern/Mediterranean sweet confectioneries made of crushed sesame seeds and syrup. [2] Folkehelsa = Norwegian Institute of Public Health, [3] RIVM = Dutch Institute for Public Health and the Environment, [4] ATCC = American Type and Culture Collection, [*] LT-2 = non-DT104 reference strain.

RESULTS

ACID RESISTANCE

Mimicking bacterial passage in a resting human stomach, bacteria were challenged for 2 hours at pH 2.5. A significant difference could be detected between human and food isolates regarding their acid resistance properties. The level of survival of *S.* Typhimurium DT104 strains exposed to pH 2.5 varied strongly (Table 2.1). Six of the *S.* Typhimurium DT104 strains were high acid resistant, thirteen DT104 strains and the reference strain BAA-118 were intermediate acid resistant and eighteen strains and the reference strain LT-2 were low acid resistant. The mean acid resistance of human non-reference strains was significantly higher after 1 hour (P=0.00092) and after 2 hours (P=0.0079) compared to food isolates using a student *t*-Test for independent samples assuming unequal variances

(Fig. 2.1). This student *t*-Test was performed on a linear scale but the same statistical analysis on a logarithmic scale gave also significant differences. In addition to the two reference strains, we selected four *S.* Typhimurium DT104 strains and used these in further experiments. Selection was based on isolation source and acid resistance. We selected two human isolates: strain 2945 (high acid resistant) and strain 911 (low acid resistant). Moreover, two food isolates were selected: strain 3633 (intermediate acid resistant) and strain 327 (low acid resistant strain).

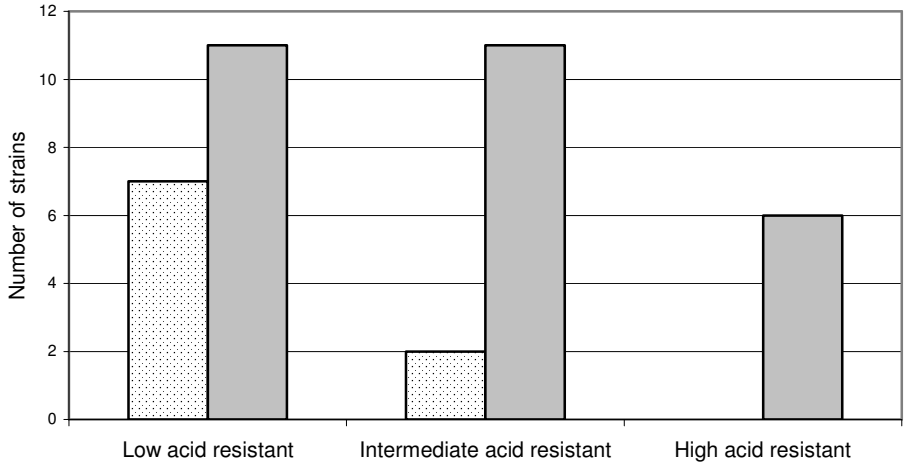

Fig. 2.1 Distribution of* S. *Typhimurium DT104 cells isolated from humans (solid bars) and foods (dotted bars) according to their acid resistance. *Total number of* S. *Typhimurium DT104 cells is 37 of which 11 were isolated from foods and 26 from humans. Statistical analysis revealed that human isolates were significantly more acid resistant (see results section).*

HEAT RESISTANCE

Heat resistance of acid-adapted stationary phase cells was determined at 55°C in LBG pH 5.0. The level of survival after 2 hours was approximately 0.035% for the *S.* Typhimurium DT104 strain, except for strains 327 and 2945 that respectively showed an small increased survival of 0.11 and 0.26% after 2 hours at 55°C. However we did not find a correlation between heat resistance and acid resistance since strain 327 was characterized as low acid resistant and strain 2945 as high acid resistant (Fig. 2.2).

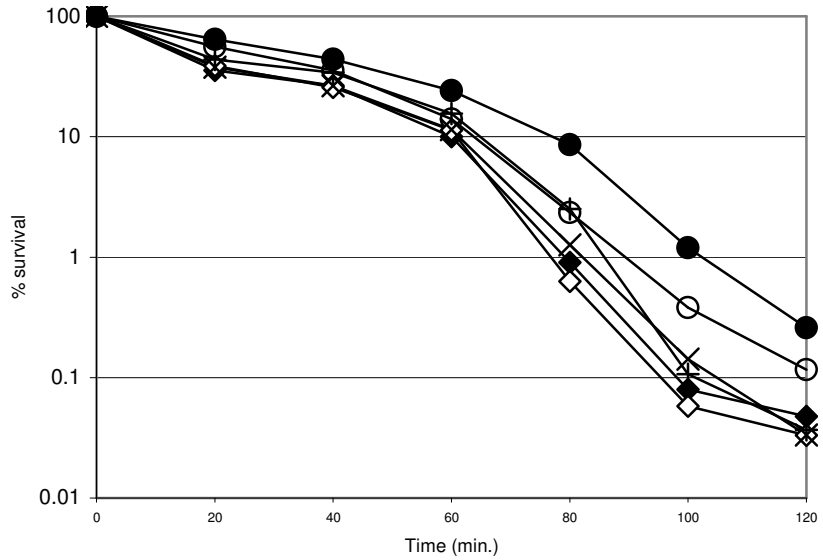

Fig. 2.2 Survival of stationary phase* S. *Typhimurium strains at 55 ℃ in LBG pH 5.0. S. *Typhimurium strain BAA-118 (×), strains LT-2 (♦), strain 327 (○), strain 911 (+), 2945 (•) and strain 3633 (◊). Heat resistance was calculated as percentage survival at t = x. HR = (cfut=x/cfut=0) × 100%.*

RPOS STATUS OF THE S. *TYPHIMURIUM DT104 STRAINS*

To determine whether mutations in the *rpoS* gene could explain the differences in acid resistance in the different *S.* Typhimurium DT104 strains we sequenced the *rpoS* gene including the promoter region of the six selected strains. Sequence analysis did not reveal any mutations in the *rpoS* gene and no differences were detected in the promoter region when compared to DT104 sequences found in the GenBank database (data not shown). Consequently, differences in acid resistance in the DT104 strains are not the result of mutations in *rpoS*. The sequence of the *rpoS* gene of *S.* Typhimurium LT2 was found to be identical to the one deposited in Genbank including its TTG startcodon, which differs from the wild type ATG startcodon. The ATG start codon is required for sustaining an acid tolerance response at low pH in exponential phase *S.* Typhimurium cells adapted at pH 4.5 [11]. Moreover, this *rpoS* allele in *S.* Typhimurium LT2 was demonstrated to be important in the avirulent phenotype of this strain [12].

PROTEIN PROFILE OF THE S. *TYPHIMURIUM DT104 STRAINS*

2D Gel electrophoresis was used to examine the protein composition of stationary phase cells grown in LBG pH 5.0 at 37°C for 18 hours. On gels containing protein extracts of *S.* Typhimurium cells, a total of approximately 200 proteins could be identified (Fig. 2.3). Detailed analysis of the 2D electrophoresis gels revealed that 5 proteins were induced differentially in *S.* Typhimurium cells (more than 1.5 times up or down regulated). The proteins FliC, FljB, DnaK, AtpB and GroEL were identified with MALDI-TOF MS and their expression levels compared to those in the reference strain *S.* Typhimurium BAA-118 were calculated (Table 2.2).

Table 2.2 Identification of protein spots by MALDITOF-MS of in-gel trypsin digested spots

Strain			BAA-118	3633	911	327	2945
Acid Resistance			Intermediate	Intermediate	Low	Low	High
Isolation source			Human	Food	Human	Food	Human
Spot	Protein	Function					
1	FliC	phase 1 flagellin	1.0	3.7	4.1	5.0	0.3
2	FljB	phase 2 flagellin	1.0	7.7	82.4	8.6	1.1
3	DnaK	heatshock chaperone	1.0	1.6	1.7	1.6	3.5
4	AtpB	ATP synthase beta chain	1.0	0.7	1.0	0.9	3.7
5	GroEL	chaperone	1.0	1.3	1.4	1.7	0.9

Spot densities are integrated and normalized to the total density in the valid spots. Fold expression of a single spot was compared to S. *Typhimurium BAA-118, which was set to 1 as a reference.*

Grouping the strains based on their acid resistance properties revealed an increased amount of the FliC and FljB proteins in the low acid resistant strains 911 and 327. The level of expression of FljB in the high acid resistant strain 2945 was similar to that in strain BAA-118 and FliC was even lower. The relative amount of flagellins in the intermediate acid resistant strain 3633 was increased. Only in the high acid resistant strain 2945 the amount of the beta chain of the ATP synthase, AtpB, was 3.7 fold higher, while in the other strains the amounts were approximately similar to that of strain BAA-118.

As shown in Fig. 2.2 the strains 327 and 2945 were slightly more heat resistant at 55 °C. The protein profiles showed that the relative amounts of the heat shock chaperones GroEL and DnaK were 1.7 and 3.7 fold higher in strain 327 and in strain 2945, respectively.

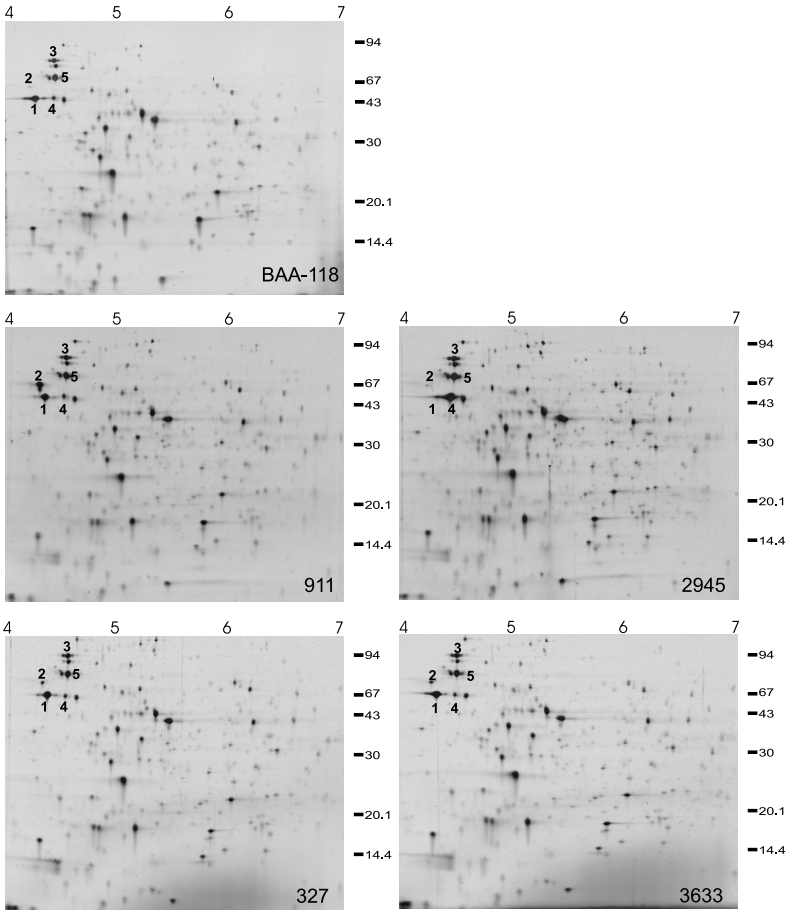

Fig. 2.3 Representative 2D electrophoresis gels of extracts of stationary phase* S. *Typhimurium cells grown in LBG pH 5.0 at 37°C for 18 h. *Molecular masses (in kDa) of the protein marker (right side) and pH ranges (top) are indicated. Proteins identified are numbered (see Table 2 2).*

DISCUSSION

Since gastric fluid is the first line of defense against microbial food borne diseases the ability of bacteria to survive in acidic environments could play a crucial role in pathogen virulence. The evolutionary benefits of high acid resistant enteric bacteria are suggested to be an improved survival during the passage of the gastro-intestinal tract and in phagosomes of the infected host. Several animal studies describe that buffering the gastric fluid resulted in an increased number of infected subjects after infection with pathogens [13;14]. In the present study we found large variations in the level of acid resistance among *Salmonella* Typhimurium DT104 strains. Interestingly, all high acid resistant strains were isolated from patients suggesting a positive correlation between acid resistance and pathogenicity. However, other factors like the food matrix play a role in pathogenicity. *S.* Typhimurium DT104 strains isolated from Helva, a mildly acidic product (pH Helva approximately 6), appeared to be low acid resistant but caused a large outbreak in June 2001 in Sweden, Norway, Australia, Germany and Turkey [15]. It seems that acid resistance properties did not play a role in this outbreak, and it is assumed that the high fat fraction in the food matrix protected the bacteria against the gastric acid in the human stomach as shown previously for enteric pathogens (Waterman and Small 1998). Notably, a number of *S.* Typhimurium DT104 outbreaks have previously been associated with foods containing a high fat fraction [16-20]. The reference strain *S.* Typhimurium BAA-188 is a human intermediate acid resistant isolate, and also here food may have served as a protective vehicle in infection.

Cells that are adapted to mild acid stress usually show cross protection to many other environmental stresses [21]. The results presented in Fig. 2 suggest that the food isolate *S.* Typhimurium DT104 327 and the human isolate *S.* Typhimurium DT104 2945 were slightly more heat resistant. Proteomics showed differences in the amount of the heat shock chaperones DnaK and GroEL in strain 2945 and 327, respectively. This may provide an explanation for the increased heat resistance. One well-known response regulator is the alternative sigma factor RpoS that plays a key role in the survival of bacteria under stress conditions [22]. Sequencing of the *rpoS* gene in the *S.* Typhimurium DT104 strains revealed no mutations or deletions. A similar observation was found by Robbe-Saule *et al.* [23] who found that the *rpoS* genes of 37 human isolates of *S.* Typhimurium were unaltered. It was suggested that mutations in *rpoS* are counter-selected, conceivably because the alternative sigma factor RpoS plays a role in pathogenesis and/or in the transmission cycle of the disease [23].

Acid resistance in *Salmonella* is described to be quite complex and approximately 60 acid shock proteins are suggested to be involved in acid resistance of which more than 10 are regulators of DNA transcription [21]. To find an explanation for the differences in acid

resistance levels in *S.* Typhimurium DT104 we examined the proteome of acid sensitive and acid resistant strains. In the low acid resistant isolates *S.* Typhimurium DT104 strain 911 and strain 327 the relative amount of the phase 1 flagellin FliC and phase 2 flagellin FljB increased. Adams *et al.* [24] suggested that flagella-mediated cell motility is co-regulated by low pH via the PhoPQ signal transduction pathway and that flagellar repression at low pH conserves ATP for survival and decreases influx of protons into the cytosol since flagella are driven directly by proton influx ([25]). Therefore, the relatively large amounts of flagellar proteins in low acid resistant strains may have contributed to poor survival at low pH. However, in the intermediate acid resistant isolate, *S.* Typhimurium DT104 3633, the expression of FliC and FljB is slightly increased, suggesting decreased acid resistance. But as can be seen in Table 2.1 strain 3633 is 10 times more acid resistant compared to the reference strain BAA-118. Obviously, cellular parameters involved in acid resistance in this strain remain to be identified.

In the human acid resistant isolate *S.* Typhimurium DT104 2945 a relatively large amount of AtpB was found, the beta chain of the H^+/ATPase in *Salmonella*. A role for the H+/ATPase in pH homeostasis in *Salmonella* has been suggested previously (Foster and Hall 1990) by studying an ATPase deletion mutant. It was found that this strain did not survive at low pH. A role for the H+/ATPase in acid resistance was also suggested in the Gram-positive *Lactococcus lactis,* in which at lower pH values (5.5 versus 7) higher levels of H+/ATPase coincided with increased acid resistance [26]. Recently, De Jonge *et al.* [8] demonstrated that growth at pH 5 compared to growth at pH 7 resulted in slightly lower amounts of H+/ATPase in a range of *S.* Typhimurium DT104 strains. In addition, no correlation was found between the amount of H+/ATPase and acid resistance properties of the individual strains. However, our proteome analysis of different *S.* Typhimurium DT104 strains showed that the acid resistant strain 2945 had an increased amount of H+/ATPase in combination with low levels of flagellar proteins. The exact role of the H+/ATPase in pH 2.5 acid resistance and the contribution of other cellular parameters to survival of *S.* Typhimurium DT104 under these conditions remain to be elucidated.

Our results with *S.* Typhimurium DT104 point to a positive correlation between acid resistance and pathogenicity as previously suggested for enteric bacteria by Bearson *et al.* [27]. The role of acid resistance in virulence will be further tested in *in vitro* studies by investigating survival in acidic macrophages and in *in vivo* studies by using a rat model allowing assessment of the survival capacities of the different DT104 isolates during gastric passage.

ACKNOWLEDGEMENTS

We would like to thank the Diagnostic Laboratory for Infectious Diseases and Prenatal Screening of the National Institute of Public Health and the Environment for PFGE analysis and Ole Alvseike of the Norwegian Institute of Public Health for supplying us with the strains. We also thank the Nutrition, Health, Safety and Sustainability program of the Netherlands Organisation for Health Research and Development for funding this research.

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CHAPTER 3

Effect of acid adaptation on *in vitro* virulence of food and clinical isolates of *Salmonella enterica* serovar Typhimurium DT104 in a fully differentiated intestinal cell line.

P.A. Berk, T. Abee and R. de Jonge

ABSTRACT

The objective of this study was to develop a virulence assay which could be used for multi-antibiotic resistant bacteria, in special *S.* Typhimurium DT104. Fully differentiated epithelial cells, Caco-2 cells, were used as a model. Furthermore the effect of different growth conditions in this virulence assay was investigated.
The original gentamicin invasion assay was used with modifications. A combination of gentamicin and ciprofloxacin was used as antibiotic to kill all extracellular bacteria. Transepithelial resistance of the Caco-2 cells was the same for cells exposed to the combination of gentamicin and ciprofloxacin as for cells exposed to gentamicin only. Adhesion and invasion of a non-gentimicin resistant *Salmonella* (Se 97-198) with the use of the combination of gentamicin and ciprofloxacin was comparable to the adhesion and invasion with gentamicin alone. We were able to determine adhesion and invasion of *S.* Typhimurium DT104 onto Caco-2 cells and we quantified the production of IL-8 by these cells in response to exposure to *S.* Typhimurium DT104. All 5 *S.* Typhimurium DT104 strains cultured at pH 7 displayed similar adhesion and invasion capacity and induced comparable levels of IL-8 produced by the Caco-2 cells. All 5 *S.* Typhimurium DT104 strains were also cultured at pH 5 to induce acid resistance. The level of invasion and adhesion, as well as the production of IL-8 was comparable for strains cultured at pH 5 and pH 7. However *S.* Typhimurium DT104 strains grown to the exponential phase showed increased invasion, but not adhesion and IL-8 production, compared to the same strains to the stationary phase.

INTRODUCTION

Salmonella enterica serovar Typhimurium DT104 (*S.* Typhimurium DT104), resistant to ampicillin, chloramphenicol, streptomycin, sulphonamide, and tetracycline (R-type 1ACSSuT), was first isolated in the UK in 1984. Since then the incidence of *S.* Typhimurium DT104 increased in many countries including the Netherlands [1]. There is a concern that in addition to resistance to antibiotics, *S.* Typhimurium DT104 strains may also exhibit increased tolerance to other antimicrobial compounds and to mild preservation treatments and processes commonly used in food production, including acidification. In a previous study we found that *S.* Typhimurium DT104 strains isolated from humans were more acid resistant than strains isolated from food products [2]. It is known that pH is an important environmental factor that influences amongst others the virulence of bacteria. *Salmonella* species can adapt to acidic conditions and therefore are of special concern in food safety. Acid adaptation protects *Salmonella* also against other sorts of stresses including high temperature, oxidative (H_2O_2) and osmotic stress [3].

To study the effect of acid adaptation on virulence *in vitro* a good *in vitro* virulence assay is needed. Several human cell lines are available to study interactions of bacteria with epithelial cells. The human adenocarcinoma cell lines, Caco-2, HT-29 and T84, have been shown to form polarized monolayers and well-defined brush borders, mimicking the human intestinal epithelium. The Caco-2 cell line is known to spontaneously differentiate to form such polarized cell layers [4]. The cells have been isolated from an adult human colon, and they express several markers characteristic of normal small intestinal villus cells [5]. The Caco-2 cell line has been shown to be a good *in vitro* system for the analysis of *Salmonella* virulence characteristics. One study [6] showed that data obtained with the Caco-2 cell line correlated well with results obtained with animal infection models for *S.* Typhimurium and *Salmonella* Cholearaesuis [7]. Also Caco-2 data for *S.* Typhimurium [8] have been shown to correlate with the rabbit ileal invasion assay. The ileal invasion assay is expected to provide solid data on virulence, since it is based on an in situ assay. *In vitro* virulence of *S.* Typhimurium DT104 has previously been determined using Hep-2 cells [9;10;10;10] and undifferentiated intestinal cells (INT-407) [11] but never in fully differentiated intestinal cells such as the Caco-2 cell line. It is expected that the latter approach mimics the *in vivo* infection pathway more closely because of the similarity of Caco-2 cells with the epithelial cells of the ileum i.e. the site of *Salmonella* invasion.

The induction of the host immune system is also a factor influencing the virulence of pathogens. Communication between epithelial cells and inflammatory cells occurs via cytokines. Epithelial cells can produce Interleukin 8 (Il-8) in response to bacterial adhesion [12] to alert the host immune system. IL-8 is a member of the chemokine superfamily stimulating migration of neutrophils, monocytes, lymphocytes and fibroblasts from the

blood to the site of infection.
This research aims to develop an assay to study different virulence parameters including adhesion and invasion, and the induction of the immune response of multi-antibiotic resistant *S.* Typhimurium DT104. This assay will than be used to study the impact of low pH growth and growth phase of *S.* Typhimurium DT104 on these virulence parameters.

MATERIALS AND METHODS

BACTERIA CULTURES, GROWTH MEDIA AND CONDITIONS

Five *S.* Typhimurium phage type 104 (ST DT104) strains and 1 *S.* Enteritidis strain were used in these experiments (Table 3.1). All strains were stored at -70°C in 50% Brain Heart Infusion (BHI, Difco) broth plus 50% glycerol. Strains were allowed to recover from –70°C for 8 hours at 37°C in BHI without shaking, after which the bacteria were used in the different experiments. Growth media used in the different experiments consisted of LB broth (10 g l^{-1} NaCl, 10 g l^{-1} trypton (Oxoid) and 5 g l^{-1} yeast extract (Oxoid)) + 4 g l^{-1} glucose (LBG), buffered with 100 mmol l^{-1} 4-morpholine-propanesulfanic acid (Mops; Roche) at pH 7 or 100 mmol l^{-1} 2-morpholine-ethanesulfanic acid monohydrate (Mes; Merck) at pH 5 in closed bottles without shaking. For stationary cells (18 hours at 37°C) OD_{660} was approximately 2 for pH 7 and 0.7 for pH 5. For exponential cells the bacteria were grown for three hours to an OD_{660} value of 0.2.

Table 3.1 **Salmonella** ***strains used in this study***

strain	type	acid resistance	isolation source	source
BAA-188	*S*T DT104	intermediate	human	ATCC*
327	*S*T DT104	low	food	Folkehelsa†
911	*S*T DT104	low	human	Folkehelsa
2945	*S*T DT104	high	human	RIVM‡
3633	*S*T DT104	high	food	RIVM
Se 97-198	*S. Enteritidis*			RIVM

* ATCC = American Type Culture Collection,
† Folkehelsa = Norwegian Institute of Public Health,
‡ RIVM = Dutch National Institute for Public Health and the Environment.

CELL CULTURES, GROWTH MEDIA AND CONDITIONS

Caco-2 cells were obtained from the American Type Culture Collection (ATCC) and passages 25-45 were used in these experiments. Cells were grown confluently at 37°C and 5% CO2 in air, in Dulbecco's modified Eagle's medium (DMEM) with 25 mM Hepes and 4.5 g l^{-1} glucose without sodiumpyruvate (GibcoBRL, Life Technologies ltd, Paisley, Scotland). Fifty ml heat inactivated (30 minutes at 60°C) fetal bovine serum (Integro b.v., Zaandam, the Netherlands), 5 ml MEM non-essential amino acids (Gibco), 5 ml L-glutamine (final concentration 6 mmol l^{-1}; Gibco) and 0.5 ml Gentamicin (final concentration 0.5 µg ml^{-1}; Gibco) was added to 500 ml DMEM. For each experiment, cells were seeded at 160.000 cells per well in 12 well tissue culture plates (surface area per well is 401 mm^2) (Costar, Corning Costar Europe, Badhoevedorp, the Netherlands). For determining the transepithelial resistance (TER) 12 well tissue culture plates with filter inserts (Costar, Corning Costar Europe, Badhoevedorp, the Netherlands) were used. Cells were grown confluently and used 12-19 days after plating; medium was changed 3 times a week. One hour before infection, medium was replaced by culture medium without serum and gentamicin, this is called tissue culture medium (TCM).

INOCULATION

S. Typhimurium DT104 strains were cultured at both pH 7 and pH 5. Each of these stationary phase culture of *Salmonella* (18 h, 37°C) was spun 10 minutes at 4500 x g. The pellet was concentrated in TCM to obtain concentrations of approximately $2.5*10^{10}$ bacteria. Each well was inoculated with 40 µl bacterial suspension, containing approximately 10^9 bacteria.

TRANSEPITHELIAL RESISTANCE (TER)

To monitor the epithelial consistence we measured the transepithelial resistance of the cell-line using a Millicell-ERS from Millipore (Billerica, Mass, USA) as described by the manufacturer. Cells were put at room temperature 10 minutes before measuring the TER. Transepithelial resistance was measured in triplicate several time points after exposure to the *S.* Typhimurium DT104 strains or addition of ciprofloxacin.

ADHESION AND INVASION STUDIES

Two hours after inoculation epithelial cells were washed 3 times with TCM. For the adhesions experiments the cells were lysed with 1 ml of 1% Triton-X100 (Merck). To study invasion two hours after infection, cells were washed 3 times with TCM and incubated an additional 3 hours with 1 ml 300 µg ml^{-1} gentamicin and 300 µg ml^{-1} ciprofloxacin in TCM.

After incubation cells were washed 3 times with TCM and also lysed with 1 ml 1% Triton-X100. Adhesion and invasion were determined by counting colony-forming units (cfu), after serial dilution in peptone buffered physiological salt (pps), on trypton soya agar (TSA)-plates. Plates were counted after 18 hours incubation at 37°C. All tests were performed five times. Data were analyzed on log scale by analysis of variance using MS Excel®. Results were considered statistically significant at $P<0.05$

IL-8 PRODUCTION BY CACO-2 CELLS

Two hours after exposure, bacterial suspensions were removed and epithelial cells were washed 3 times using TCM. One ml 300 µg ml^{-1} gentamicin and 300 µg ml^{-1} ciprofloxacin in TCM was used to kill all external bacteria. After 3 hours of incubation the medium was replaced by TCM. Samples were taken 24 hours after infection and stored at -70°C to be analysed later. Caco-2 cells without bacteria were used as control. All tests were performed in triplicate.

IL-8 concentrations were determined using an IL-8 ELISA according to Garssen et al. [13]. Briefly, 96-well plates (NUNC-Immuno Plate, Roskilde, Denmark) were coated with 1 µg ml^{-1} antihuman IL-8 (Biosource, Nivelles, Belgium) in coating buffer (0.04 mol l^{-1} carbonate buffer, pH 9.6). After 24 hours incubation at 4°C, the plates were incubated in blocking buffer, 1% Bovine Serum Albumin (BSA; Sigma, Axel, the Netherlands) plus 0.05% Tween-20 (Merck, Amsterdam, the Netherlands) in phosphate buffered saline (PBS) during 24 hours at 4°C and washed in PBS with 0.05% Tween-20. Recombinant human IL-8 was used as a standard. Standard, as well as serial dilutions of culture supernatant and 0.2 µg ml^{-1} biotinylated antihuman IL-8 (Medgenix diagnostics SA, Fleurus, Belgium) was added to the plates. After incubation for 2 hours at 21°C plates were washed and 0.1 µg ml^{-1} poly horseradish peroxidase-labelled streptavidin (Central Laboratory of the Blood Transfusion Service, Amsterdam, the Netherlands) was added. After incubation for 30 minutes at 21°C plates were washed again and 0.1 mg ml^{-1} tetra methyl benzidine (TMB) (Sigma Chemical Co, St. Louis, MO) plus 0.006% H_2O_2 in 0.1 mol l^{-1} Na-acetate, pH 5.5 was added. The colour-reaction was stopped after 5 minutes by adding 50 µl 1 mol l^{-1} H_2SO_2 (Merck). Plates were read at 450 nm using a Titertek Multiskan MCC/340 ELISA reader. Data were analyzed by analysis of variance using MS Excel®. Results were considered statistically significant at $P<0.05$

RESULTS

TRANSEPITHELIAL ELECTRICAL RESISTANCE (TER)

Since most *S.* Typhimurium DT104 strains are multi-antibiotic resistance, an original gentamicin invasion assay could not be used. Therefore an antibiotic had to be found that kills all the external *S.* Tyhpimurium DT104 strains. 10^8 cfu was added to tissue culture medium with different concentrations and combinations of different antibiotics. Only the combination of 300 µg/ml gentamicin and 300 µg/ml ciprofloxacin killed all strains tested completely in 3 hours. Since ciprofloxacin is known for its intracellular functioning it was also tested whether ciprofloxacin affects Caco-2 cells. The transepithelial resistance (TER) of Caco-2 cells was followed for 3 hours in the presence of 300 µg/ml gentamicin and 300 µg/ml ciprofloxacin and compared to that of the TER of Caco-2 cells incubated in tissue culture medium. Notably no differences in resistance were found (Fig. 3.1). In an additional control experiment the amount of invasion of a non-gentamicin resistant *Salmonella* (Se 97-198) in presence of 300 µg/ml gentamicin and 300 µg/ml ciprofloxacin was compared to the amount of invasion in the original invasion assay with only gentamicin. Again similar results were obtained (Fig. 3.2). Therefore is was concluded that the invasion assay for the *S.* Typhimurium DT104 strains can be conducted with 300 µg/ml gentamicin and 300 µg/ml ciprofloxacin without affecting the Caco-2 cells or intracellular *Salmonella*.

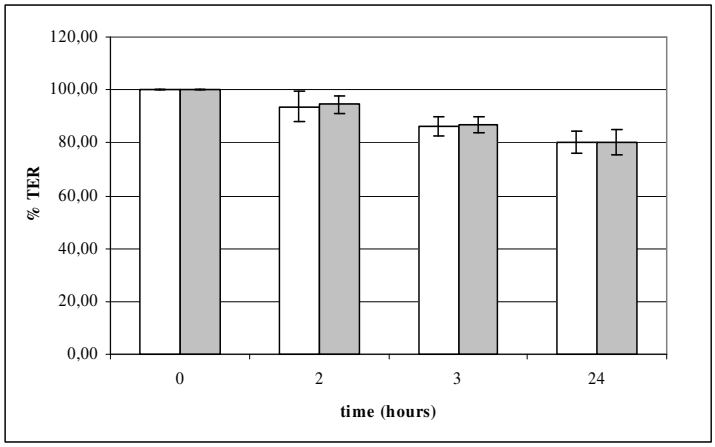

Figure 3.1. Transepithelial resistance of Caco-2 cells in the presence and absence of ciprofloxacin. *Caco-2 cells were exposed to TCM with 300 µg/ml ciprofloxacin and 300 µg/ml gentamicin (closed bars) or to TCM without additions (open bars). Values represent mean percentage of TER in relation to the start TER and standard deviation of values of 6 experiments.*

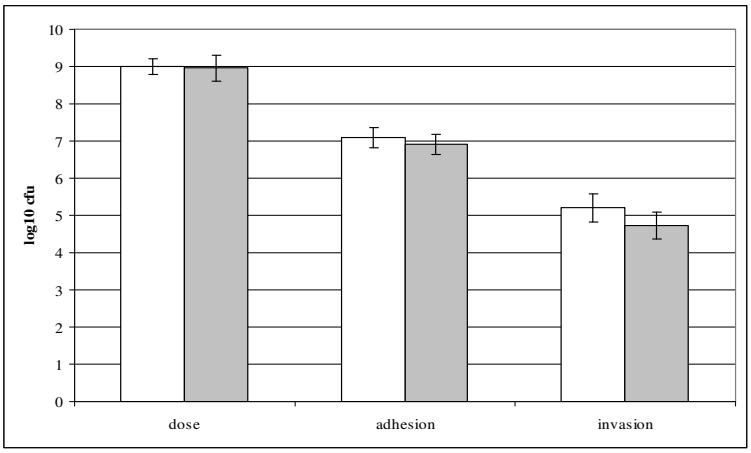

Figure 3.2. Interaction of* S. *Enteritidis 97-198 strains with Caco-2 cells. S. *Enteritidis 97-198 was used to infect a Caco-2 monolayer with approximately 10^9 cfu. Two hours after inoculation the amount of bacteria attached to the cells and bacteria invaded were determined. For the invasion experiments two hours after infection the bacteria were removed and extracellular bacteria were killed in 3 hours using gentamicin only (open bars) or a combination of 300 μg/ml ciprofloxacin and 300 μg/ml gentamicin (closed bars). Values represent means and standard deviation of log transformed values of at least 15 experiments.*

VIRULENCE ASSESSMENT OF DIFFERENT S. TYPHIMURIUM DT104 ISOLATES

In vitro virulence parameters were determined using five *S.* Typhimurium DT104 strains grown at pH 7. The Caco-2 cells were infected with approximately 10^9 bacteria. All strains adhered well to the Caco-2 cells (Fig. 3.3a). No significant differences could be found between the different strains (p=0.06) using one way ANOVA. Furthermore, all *S.* Typhimurium DT104 strains were able to invade the Caco-2 cells with similar invasion capacity (Fig. 3.3b) (p=0.19). In addition, IL-8 production was measured 24 hours after infection with *S*. Typhimurium DT104 (Fig 3.3c). Also no significant differences could be found with regard to their ability to induce IL-8 production by Caco-2 cells (p=0.10).

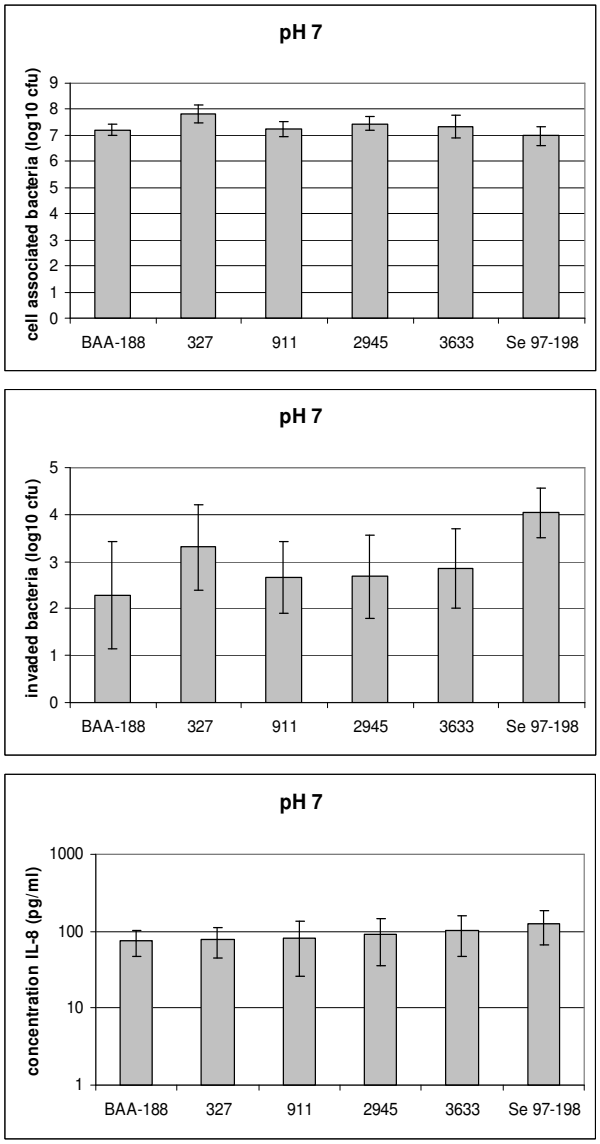

Figure 3.3. Interaction of* S. *Typhimurium DT104 strains grown at pH 7 with Caco-2 cells. Different* S. *Typhimurium DT104 strains and one* S. *Enteritidis strain were cultured at pH 7 and used to infect the Caco-2 monolayer with approximately 10^9 cfu. Two hours after inoculation the amount of bacteria attached to the cells (including invaders) was determined. Values represent means and standard deviation of log transformed values of at least 5 experiments (A). Two hours after infection the bacteria were removed and extracellular bacteria were killed in 3 hours using a combination of 300 μg/ml ciprofloxacin and 300 μg/ml gentamicin. Values represent means and standard deviation of log transformed values of at least 6 experiments (B). IL-8 was measured 24 hours after infection with S. Typhimurium DT104 in the supernatant of the Caco-2 cells. Non-infected Caco-2 cells were used as a control. Values represent means minus blanc and standard deviation of values of 7 experiments (C).

LOW PH GROWTH AND VIRULENCE

To determine whether growth at low pH affects virulence of *S.* Typhimurium DT104 strains, the selected strains were grown at pH 5. Notably, no significant differences could be found between the different strains when cultured under acid-adaptive conditions with regard to their adhesion (p=0.59) and invasion (p=0.06) capacity, and induced comparable levels of IL-8 production by Caco-2 cells (Fig. 3.4a, 3.4b and 3.4c) (p=0.65). A comparison of the virulence parameters obtained with the *S.* Typhimurium DT104 strains grown at pH 5 and pH 7 reveals that they are highly similar, indicating that the pH of the growth medium does not affect the in vitro determined virulence characteristics.

GROWTH PHASE AND VIRULENCE

Two *S.* Typhimurium DT104 strains were cultured at pH 7 and grown to the exponential phase for 3 hours to determine whether growth phase affects the virulence of *S.* Typhimurium DT104 strains. Data from bacteria grown to the exponential phase were compared to bacteria grown to the stationary phase using a student's t-test. No significant differences could be found between the different strains when cultured to the exponential phase compared to the stationary phase with regard to their adhesion (911: p=0.67, 2945: p=0.10) and the induction of IL-8 production by Caco-2 cells (911: p=0.07, 2945: p=0.43). However, the invasive capacity was clearly increased for cells grown to the exponential phase compared to the stationary phase (Fig. 3.5a, 3.5b and 3.5c) (911: p=0.0009, 2945: p=0.003).

DISCUSSION

In this study the in vitro virulence of *S.* Typhimurium DT104 was tested for the first time in a Caco-2 cell line, consisting of fully differentiated intestinal cells. We were able to determine adhesion and invasion of *S.* Typhimurium DT104 onto the Caco-2 cell line, and the production of IL-8 by the Caco-2 cells in response to *S.* Typhimurium DT104 infection. Since gentamicin resistance is found in multiresistant *S.* Typhimurium DT104, invasion assays were performed using high concentrations of ciprofloxacin in combination with gentamicin, which killed the bacteria, but did not affect the TER of Caco-2 cells or the invaded *Salmonella.*

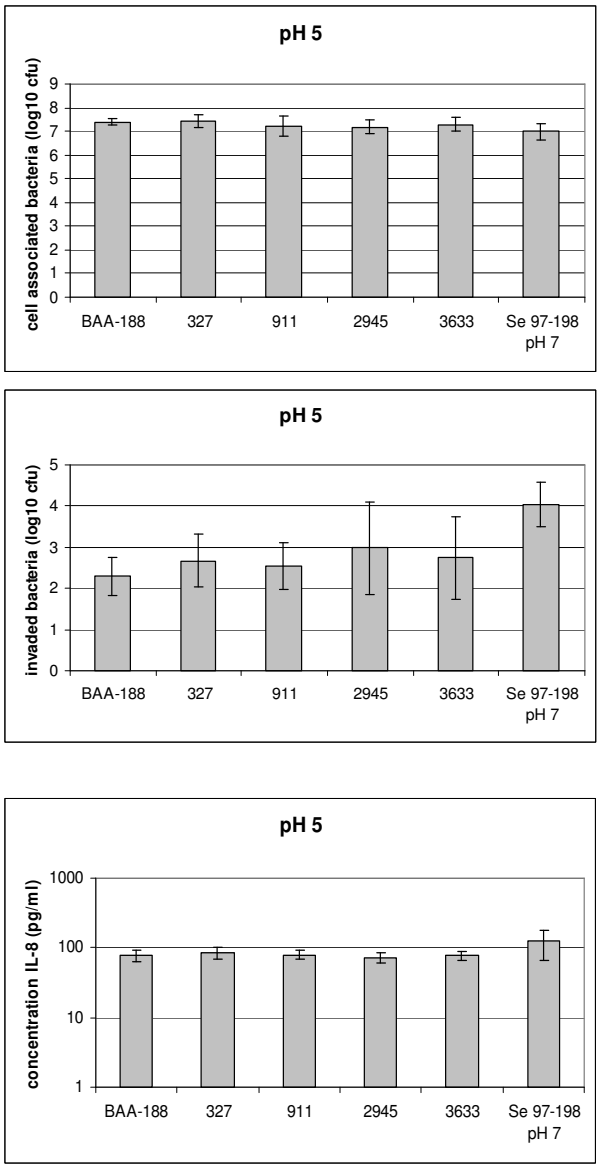

Figure 3.4. Interaction of* S. *Typhimurium DT104 strains grown at pH 5 with Caco-2 cells. *Different* S. *Typhimurium DT104 strains were cultured at pH 5 and used to infect the Caco-2 monolayer with approximately* 10^9 *cfu.* S. *Enteritidis was cultured at pH 7 and used as an internal standard of the experiments. Two hours after infection the amount of bacteria attached to the cells was determined. Values represent means of adhered* Salmonella *and standard deviation of log transformed values of at least 5 experiments (A). Two hours after infection the bacteria were removed and extracellular bacteria were killed in 3 hours using a combination of 300 µg/ml ciprofloxacin and 300 µg/ml gentamicin. Values represent means of invaded* Salmonella *and standard deviation of log transformed values of at least 6 experiments (B). IL-8 was measured 24 hours after infection with* S. *Typhimurium DT104 in the supernatant of the Caco-2 cells. Non-infected Caco-2 cells were used as blanc. Values represent means minus blanc and standard deviation of values of 4 experiments (C).*

In addition to the assessment of the adhesion and invasion capacity, the induction of a host immune response may also be an important virulence parameter. Communication between epithelial cells and inflammatory cells occurs via cytokines. One important cytokine in this respect is IL-8 which is produced constitutively at low levels by the intestinal cells [12]. The main role for IL-8 is the recruitment of neutrophils to the subepithelial space [14] in response to an infection. As a consequence IL-8 production will be increased after infection with pathogenic bacteria like *S.* Enteritidis, *S.* Dublin, *Listeria monocytogenes, Yersinia enterocolitica, Shigella dysenteriae* and *Pseudomonas aeruginosa* [12;15]. Our results revealed that the Caco-2 cells produced small amounts of IL-8, whereas subsequent infection with *S.* Typhimurium DT104 resulted in significantly increased levels of IL-8. However, the IL-8 response of the Caco-2 cells was similar for all strains grown at pH 5 and pH 7, which is in agreement with the adhesion and invasion results.

The Caco-2 model was used to study the *in vitro* virulence parameters using 5 strains of *S.* Typhimurium DT104 and 1 *S.* Enteritidis. In addition, the effect of bacterial growth conditions of low pH and growth phase, on the in vitro virulence of *S.* Typhimurium DT104 was studied. The correlation between invasion and acid survival was investigated since both are important factors in *Salmonella* pathogenesis. Although acid survival is known to be important in gastric passage and for the survival inside macrophages, little is known about the effect of low pH growth on pathogen adhesion and invasion of epithelial cells. Our results show that *in vitro* virulence characteristics of all strains of *S.* Typhimurium DT104 grown at pH 5 and pH 7 are similar. In contrast to another study [11], in our study no differences in invasion could be observed between acid adapted (pH 5) and non-adapted (pH 7) bacteria. Their results may be explained by a glucose-mediated effect, since virulence parameters of bacteria cultured in media of pH 5 with glucose were compared to that of bacteria cultured at pH 7 without glucose. Also the use of gentamicin in that study is questioned since most *S.* Typhimurium DT104 strains are resistant to gentamicin, resulting in an overestimation of the number of presumably invaded bacteria. The differences between the two studies can also be due to the use of a different cell line or to strain differences among *S.* Typhimurium DT104 strains. However since all *S.* Typhimurium DT104 strains acted similarly in this study as well as in the study of Allen *et al.*[10] the latter is not likely.

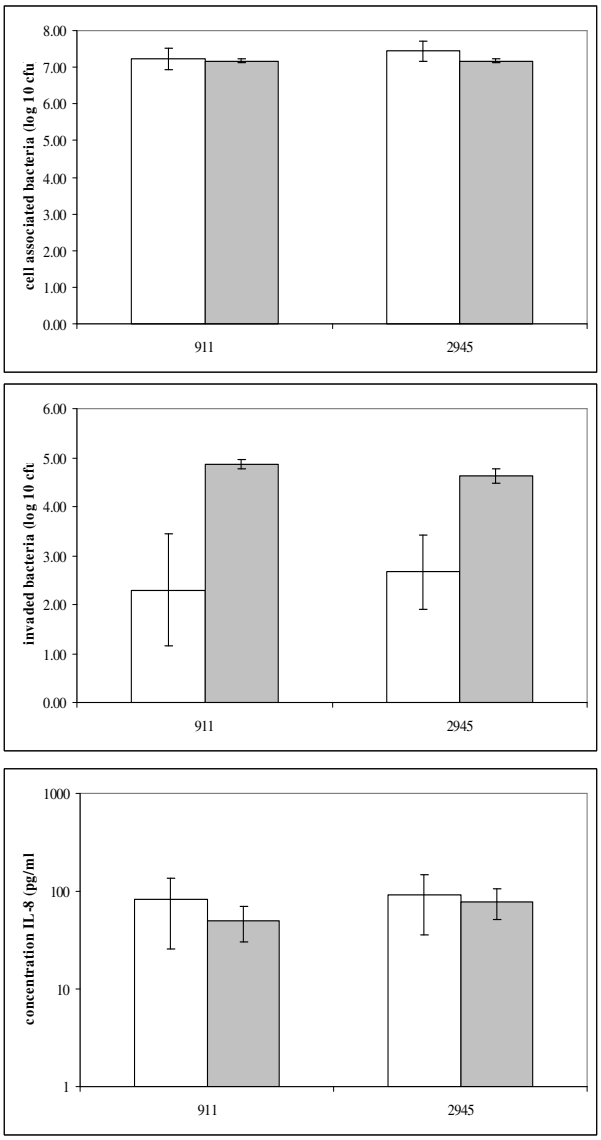

Figure 3.5.* S. *Typhimurium DT104 strains grown to the stationary (open bars) and exponential (closed bars) phase with Caco-2 cells. *Two* S. *Typhimurium DT104 strains were cultured at pH 7 to the exponential and stationary phase and used to infect the Caco-2 monolayer with approximately 10^9 cfu. Values represent means of adhered* Salmonella *and standard deviation of log transformed values of at least 5 experiments (A). Values represent means of invaded* Salmonella *and standard deviation of log transformed values of at least 6 experiments (B). Values represent means minus blanc and standard deviation of values of 4 experiments (C).*

S. Typhimurium DT104 grown to the exponential phase showed a higher invasive capacity then grown to the stationary phase. This is consistent with other *in vitro* studies that also revealed increased virulence of *Salmonella* exponential phase cells i.e. their invasion capacity was higher than that of stationary phase cells [16;17] . Hermans *et al*. [18] found in microarray studies using *S.* Typhimurium DT104 grown to different growth phases, that genes of the *Salmonella* Pathogenicity Island 1 (SPI-1) were upregulated at the end of the exponential phase. Several SPI-1 effector proteins are known to be involved in the invasion process of *Salmonella* (reviewed in [19]), therefore upregulation of SPI-1 genes can result in increased invasion and thus increased virulence. In our study no effect of growth phase was found in the adhesive capacity of *Salmonella*. However, Hermans *et al.*[18] have shown that LPS genes, which are thought to be important in the adhesion process, are also upregulated during exponential growth.

Combining these results it can be speculated that, although there can be an effect on the survival of the stomach, the low pH of certain acidic food products in which *S.* Typhimurium DT104 can be found, does not contribute to the adhesive and invasive capacities of the bacteria in the intestine. Her, the growth phase seems to be a prominent factor determining *S.* Typhimurium DT104 virulence.

ACKNOWLEDGEMENTS

We thank Prof. Dr. Ir. M. H. Zwietering for critically reading the manuscript and the Nutrition, Health, Safety and Sustainability program of the Netherlands Organisation for Health Research and Development for funding this research.

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CHAPTER 4

In vivo virulence of *Salmonella* Typhimurium DT104 in a rat model and the effect of growth pH and growth phase

P.A. Berk, T. Abee, R. de Jonge and M.H. Zwietering

ABSTRACT

The aim of this study was to determine the *in vivo* virulence of *Salmonella* enterica serovar Typhimurium phage type DT104 (*S.* Typhimurium DT104) for the first time in a rat model. Adult male Wistar-Unilever rats were exposed orally after overnight starvation to increasing doses of different strains of *S.* Typhimurium DT104. The microbiological data for the probability of positive animals on day 6 showed a regularly increasing trend with the dose and could be fitted to the exponential model, as did the data for the spleen, ileum and Peyers patches. Bacterial counts of the spleen when infected were usually between 10^3 and 10^4cfu/g, irrespective of the dose. Bacterial counts of Peyers patches were usually between 10^5 and 10^6cfu/g, but lower counts could be observed. The count of the total white blood cell populations showed also a dose dependent increase on day 6. The increase of white blood cells with increasing dose was mainly due to neutrophils and macrophages. Basophils were only slightly upregulated, whereas total counts of eosinophils and lymphocytes did not change. The effect of growth pH and growth phase was studied in this rat model, since these are known to affect the *in vitro* virulence of *Salmonella*. Growth at pH 5 instead of pH 7 and neutralisation of the stomach had no significant effect on the microbiological and haematological parameters whereas growth phase did have a large effect. The infectious dose (ID_{50}) of *S.* Typhimurium DT104 cells grown to the exponential phase appeared to be even 30-60 times lower than that of cells grown to the stationary phase. Moreover, with exponential phase cells, a large increase in microbiological counts in the spleen and Peyers patches and in neutrophils and monocytes were already observed at day 2 while with cells from the stationary phase this was only observed at day 6. Thus growth phase seems to have a large effect on the virulence of *S.* Typhimurium DT104 whereas growth pH has not.

INTRODUCTION

Infections caused by the *Salmonella enterica* serovars Enteritidis (*S.* Enteritidis) and Typhimurium (*S.* Typhimurium) continue to be a major health problem. They usually occur after consumption of contaminated food or water and can manifest in a variety of disease states, ranging from asymptomatic carrier status through gastroenteritis or even severe systemic infection that can occasionally lead to death. The nature and severity of the infections vary dependent on host, food matrix and bacterial strain involved.
S. Typhimurium DT104, resistant to ampicillin, chloramphenicol, streptomycin, sulphonamide, and tetracycline (R-type ACSSuT), was first isolated in the UK in 1984. Since then the incidence of *S.* Typhimurium DT104 increased in many countries including the Netherlands [1;2]. Some studies showed that the clinical symptoms of multi-antibiotic resistant *S.* Typhimurium DT104 were more severe than other *S.* Typhimurium or *S.* Enteritidis infections [3;4] and due to their antibiotic resistance difficult to treat. Several studies indicated that an infection with *Salmonellae* resistant to one or more antimicrobial drugs is associated with increased risk for hospitalization, invasive illness and death [5-11]. Although another study showed that the incidence of multiresistant *S.* Typhimurium DT104 from blood culture in human beings was not significantly greater than that of other *S.* Typhimurium phage types[12]. Because of the severity of disease caused by multi-antibiotic resistant *S.* Typhimurium phage type DT104 (*S.* Typhimurium DT104) and the increasing frequency of isolation of this *Salmonella* strain, *S.* Typhimurium DT104 has been proposed to be more virulent than other *S.* Typhimurium strains.

Experimental *Salmonella* infections are extensively studied in rodents. In susceptible mice such infections can cause non-typhoid *Salmonella*-induced systemic disease. Stecher *et al.* [13] described a new mouse model with predominantly intestinal disease. In rats, like in humans, generally only self-limiting gastroenteritis is observed, with only rare occasional bacteraemia. Within 2 hours after oral exposure of rats to *Salmonella*, the bacteria can be detected in the distal ileum and coecum. Invasion occurs via the M-cells in the Peyers patches and *Salmonella* can be detected in the mesenteric lymph nodes within 8 hours after infection [14]. Rats develop clinical disease only at doses higher than 10^8 colony-forming units (cfu) when administered orally [15;16].
The rodent is particularly useful for investigating the initial stages of the pathological pathway. These initial stages contain the invasion of the epithelium and the innate immune response.
The innate immune response is the first defence mechanism against intra- and extracellular bacteria. The principal mechanism of the innate immune system is phagocytosis by monocytes, tissue macrophages and polymorphonuclear leucocytes (mainly neutrophils).

Virulent *Salmonella* have adapted to survive and grow inside macrophages, and may induce cell death by apoptosis [17]. Host specific virulence may correspond to *in vitro* differential persistence in murine and human macrophages [18]. It was also found that the number of circulating neutrophils increases considerably after exposure to *Salmonella* Dublin, Enteritidis and Typhimurium[19-22]. Since *Salmonella* is a food borne pathogen, most infections occur orally. Although there is a lot of information into the pathogenesis and host responses to a *Salmonella* infection, most studies are performed using a single high dose, which is often intraperitoneally or intravenously administered. Moreover, such data are lacking for *S.* Typhimurium DT104. Recently it was shown that the rat model is a sensitive and reproducible tool for studying the effects of oral exposure to *S.* Enteritidis over a wide dose range [22]. Therefore this model was used to investigate for the first time, the virulence of different *S.* Typhimurium DT104 strains. Different factors play a role in the virulence of *S.* Typhimurium, including environmental factors, bacterial factors and host factors. First, an ATCC strain of *S.* Typhimurium DT104 (BAA-188) was used to obtain a dose response standard, to which al other experiments can be compared. In all following experiments this DT104 strain was used as an internal control allowing comparison with the standard experiment. The reproducibility of the rat model was next investigated. Then two different *S.* Typhimurium DT104 strains, isolated from patients, were compared to the standard obtained in the first experiment. *In vitro* studies revealed pH to affect the invasiveness of *Salmonella* [23] therefore the effect of low growth pH was investigated in the rat model. Since the acidic pH of the stomach is known to be important in the defence against food borne pathogens also the effect of stomach passage was investigated. Finally the effect of growth phase on DT104 *in vivo* virulence was assessed, since the ability of *Salmonella* to enter mammalian cells *in vitro* was previously shown to be affected by different phases of growth [24;25].

MATERIALS AND METHODS

ANIMALS

Specific pathogen-free (SPF) male Wistar-Unilever (WU) rats were obtained from the breeding colony at the National Institute for Public Health and the Environment, Bilthoven, the Netherlands. The animals, 6-9 weeks of age, were housed individually in macrolon cages, 2 weeks prior to inoculation. Drinking water and conventional diet (RMH-B, Hope Farms BV, Woerden, the Netherlands) was provided *ad libitum*. The breeding colony was screened for endogenous pathogenic viruses and bacteria and was found negative.

BACTERIAL STRAINS

Salmonella enterica serovar Typhimurium DT104 strain ATCC BAA-188, 911 and 2945 were used [26;27]. Non-pathogenic *Escherichia coli* WG5, a nalidixic acid-resistant derivative of *Escherichia coli* C [28], was used as a negative control. All strains were stored at -70°C in Brain Heart Infusion broth (BHI) plus 50% glycerol. Strains were allowed to recover from –70°C for 8 hours at 37°C in BHI without shaking, after which the bacteria were used in the different experiments. Bacteria were cultured in Luria Bertani broth (LB) + 4 g/l glucose (LBG), buffered with 100 mM 4-morpholine-propanesulfanic acid (Mops; Roche) at pH 7 or 100 mM 2-morpholine-ethanesulfanic acid monohydrate (Mes; Merck) at pH 5 in closed bottles without shaking. For stationary cells (18 hours at 37°C) OD_{660} was approximately 2 for pH 7 and 0.7 for pH 5. For exponential cells the bacteria were grown for three hours to an OD_{660} value of 0.2. After incubation 50 ml of each culture was centrifuged at 5000 x *g* for 10 minutes at 4°C. The supernatant was discarded and the pellet was re-suspended in 50 ml physiological saline (PS) (9 g l^{-1} NaCl), followed by re-centrifugation. The supernatant was discarded and the pellet was finally re-suspended in 5 ml PS. The bacterial suspensions were serially diluted in PS and mixed with the same volume of 6% (w/v) $NaHCO_3$ (except for the experiment where stomach passage was assessed) directly before administration to exclude any pH-effect in the stomach. Before the administration, colony forming units (cfu) were determined, after serial dilution in pepton (1 g l^{-1}) buffered physiological saline (pps), on trypton soy agar plates (TSA) after 20±2 hours incubation at 37°C.

HAEMATOLOGY

As an indicator for (systemic) infection, haematology for each rat was determined on blood samples obtained 14 days prior to infection and 2 or 6 days after infection, anticoagulated (with K_3EDTA) in 3.6 mg EDTA-tubes (Becton Dickinson Vacutainer Systems, Plymouth, United Kingdom). The haematological analyses were performed using the H1-E multi-species haematology analyser (Bayer B.B., Mijdrecht, the Netherlands) with multi-species software, version 3.0. The following parameters were determined: haemoglobin concentration; haematocrit red blood cell concentration; mean corpuscular volume; mean cell haemoglobin; mean cell haemoglobin concentration; red blood cell distribution width; haemoglobin distribution width; platelets; mean platelet volume; white blood cell concentration; differentiation of white blood cells (% and absolute numbers) into neutrophils, lymphocytes, monocytes, eosinophils, basophils and large unstained cells (LUCs).

EXPERIMENTAL DESIGN

The Central Animal Laboratory of RIVM possesses a licence under the Dutch 'Animal Experiments Act'. In accordance with Section 14 of this Act, an officer was appointed to supervise the welfare of laboratory animals. All experiments were discussed and approved by an independent ethical committee prior to the study. Blood samples were taken via orbita plexus puncture, using a capillary under light ether anaesthesia, approximately 14 days before oral inoculation to determine blood basal parameters for each animal. After 2 weeks of acclimatisation the animals were starved overnight (water *ad libitum*). After approximately 16 hours of starvation, 1 ml of the bacterial suspensions was orally administered by gavage. One group consisting of four or eight rats was infected with the same dose. Directly after gavage (day 0), food and water was provided *ad libitum*. The animals were weighed each day, starting one day prior to inoculation and daily clinical observations were made on the general health of the animals. The animals were sacrificed on day 2 or 6 after oral inoculation, by bleeding from the abdominal aorta under KRA anaesthesia (intramuscular injection of 100 µl of a cocktail consisting of 7 ml ketalar (50 mg ml^{-1}, Parke Davis, Spain), 3 ml rompun (20 mg ml^{-1}, Bayer, Leverkusen, Germany) and 1 ml of atropin (1 mg ml^{-1}, OPG, Utrecht, the Netherlands)). Approximately 1 ml of blood was obtained via orbita plexus puncture to be used for determination of haematological parameters. The spleen, the ileum, a part of the jejunum and the four most distal Peyers patches were removed aseptically. The spleen was weighed and for all organs colony forming units were determined.

MICROBIOLOGY

Internal organs were homogenised in pps using an Ultra Turrax (Janke und Kankel, Breisgau, Germany). Appropriate 10-fold dilutions in phosphate buffered PS (0.06 M) were spread-plated on brilliant green agar (BGA) for *Salmonella* Typhimurium DT104 and tryptone yeast extract glucose agar with 100 µg ml^{-1} nalidixic acid (TYGnal) for *E. coli* WG5. The BGA and TYGnal plates were incubated at 37°C for 20±2 hours.

STATISTICAL ANALYSIS

Dose-response modelling was based on the following assumptions [29]: (1) single hit, i.e. one single surviving organism is capable of initiating an infection [30]; (2) independent action, i.e. the probability of one organism initiating infection is independent of the presence of other organisms [31]; and (3) random distribution of the organisms in the inoculum. In this article, infection is detected by isolation of *Salmonellae* from the spleen, the Peyers patches, the ileum or a small part of the jejunum. If it is assumed that the probability of any organism in

any host to survive and initiate infection has a constant value *r*, then the exponential model follows:

$$P_{\text{inf}} = 1 - e^{-rD}$$

where P_{inf} is the probability of infection and *D* is the mean dose and *r* is the probability of one *Salmonella* to infect the host. If *r* is assumed to follow a Beta-distribution with parameters α and β, the hypergeometric model follows [32]. If $\alpha << \beta$ and $\beta >> 1$, this can be simplified to the Beta-Poisson model:

$P_{inf} = 1 - (1 + D/\beta)^{-\alpha}$

The models were fitted to pooled data on infection using TableCurve 2D. If there were no large differences (the differences in the coefficient of determination r^2 is less than 0.02) between the 2 models the exponential model was preferred. ID_{50} values were calculated as:

$ID_{50} = \ln(2)/r$

for the exponential model and

$ID_{50} = \beta * (2^{1/\alpha} - 1)$

for the Beta-Poisson model.

RESULTS

RAT MODEL AND REPRODUCIBILITY

To obtain a standard dose response curve, rats were inoculated with different doses of *S.* Typhimurium DT104 ATCC strain BAA-188 grown at pH 7 to the stationary phase. Both spleen weight and spleen/body weight ratio increased with increasing dose (data not shown). The results of microbiological examination of the spleen, ileum and Peyers patches (pp) or positive animals at day 6 are shown in Figure 4.1. Bacterial counts of the spleen were between 10^3 and 10^4cfu g^{-1} in *Salmonella* positives rats irrespective of dose. Bacterial counts of positive Peyers patches were between 10^5 and 10^6cfu g^{-1}, but lower counts were also observed. The data for the probability of infection in the spleen and Peyers patches and

positive animals on day 6 showed a regularly increasing trend with the dose and the exponential model could be fitted to the data ($r = 8.36 \times 10^{-5}$ for positive animals, $r = 5.02 \times 10^{-5}$ for spleen, $r = 1.19 \times 10^{-5}$ for ileum and $r = 3.31 \times 10^{-5}$ for pp). From these models, the ID_{50} is 8.29×10^{3} cfu for at least one organ infected, 1.38×10^{4} cfu for the spleen, 5.82×10^{4} cfu for the ileum and 2.09×10^{4} cfu for the Peyers patches.

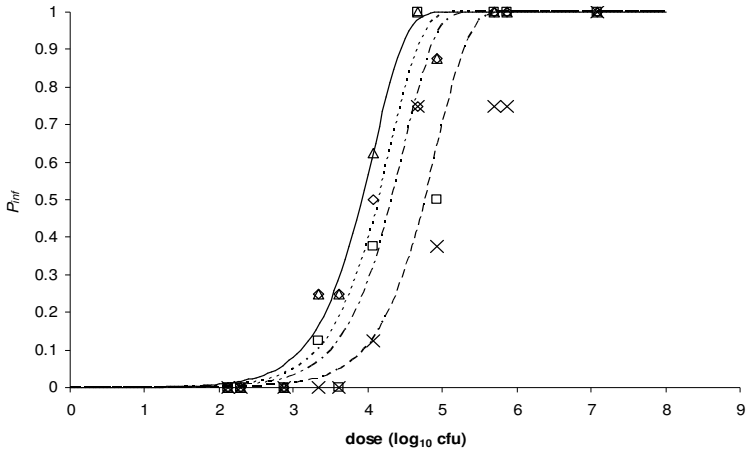

Figure 4.1. Dose response data of* Salmonella *Typhimurium DT104 ATCC strain BAA-188 in spleen (◊), ileum (x), Peyers patches (□) or positive animals (Δ) of adult male WU rats six days after oral exposure to different doses, and fitted dose-response models. *(—) positive animals exponential model, (--) spleen exponential model, (— – —) Peyers patches exponential model, (– –) ileum exponential model. P_{inf} is probability of infection.*

A new set of rats was used to test the reproducibility of the rat model. Although the variability was somewhat larger in the second experiment, the microbiological data of the second experiment were comparable to that of the first experiment (Fig. 4.2).
Besides microbiological effects also an effect on white blood cell population was observed. Six days after oral exposure to *S.* Typhimurium DT104 BAA-188 a significant dose-dependent increase in the white blood cell (WBC) population of the rats was found, whereas after day 2 no significant differences were found compared to the basic level determined 14 days before infection ($p = 0.005$ *vs* $p = 0.19$ using a paired t-test)(Fig. 4.3).

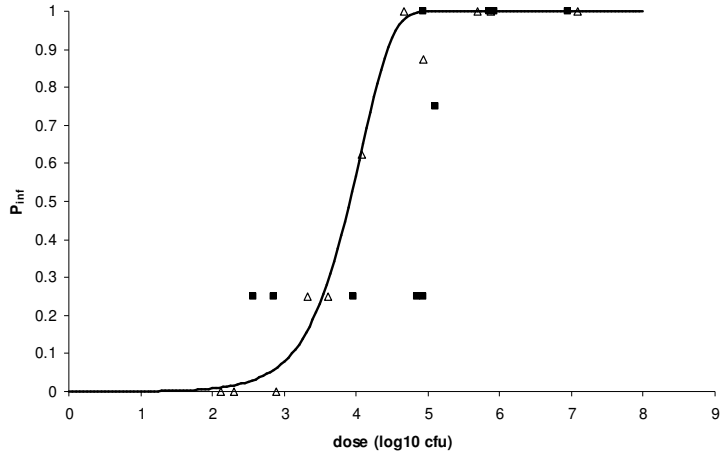

Figure 4.2. Reproducibility of infection of WU rats with* S. *Typhimurium DT104 strain BAA-188 for positive WU rats six days after oral exposure. *Shown are the data of the second experiment (■) compared to the data (Δ) and the fitted exponential model (—) of the first experiment.*

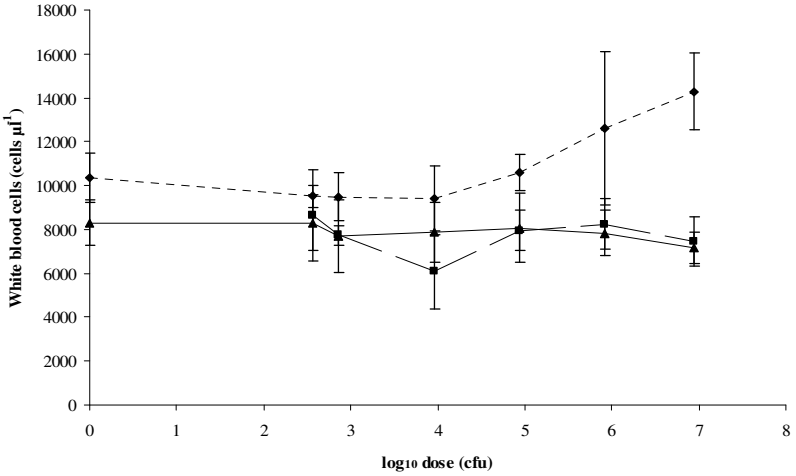

Figure 4.3. Dose-response relationship of white blood-cell counts in adult, male WU rats before and after exposure to* Salmonella *Typhimurium DT104 ATCC strain BAA-188*. Before exposure (▲), two days after exposure (■), six days after exposure (♦). Log dose 0 is the negative control group infected with* E. coli *WG5 (dose 5.62*10^6).***

Also the composition of the WBC population changed. Figure 4.4 shows the changes in the absolute numbers of the different subclasses of the white blood cell population as a function of the inoculum dose. Large increases were seen in counts of neutrophils, monocytes, basophils and large unstained cells. Lymphocyte and eosinophils counts did not change during the experiment. The large error bars found in most experiments were related to variability in white blood cell parameters between different animals within one dose group.

IMPACT OF STRAIN DIVERSITY

Different strains of *S.* Typhimurium DT104 can have different virulence capabilities. Therefore next to *S.* Typhimurium DT104 BAA-188, *S*. Typhimurium DT104 strain 2945 (acid resistant) and 911 (acid sensitive) [26]were used to study their virulence in the rat model. The strains were grown at pH 7 to the stationary phase. The results of microbiological examination of positive animals are shown in comparison to the first experiment with BAA-188. Again the data for the probability of positive animals on day 6 showed a regularly increasing trend with the dose and the exponential model could be fitted to the data ($r = 4.63 \times 10^{-5}$ for strain 2945, $r = 6.35 \times 10^{-5}$ for strain 911). The calculated ID_{50} are 1.50×10^{4} and 1.09×10^{4} for DT104 2945 and 911, respectively (Fig. 4.5). In this experiment, similar changes in white blood cell population on day 6 were found as in the first experiment with BAA-188 (data not shown).

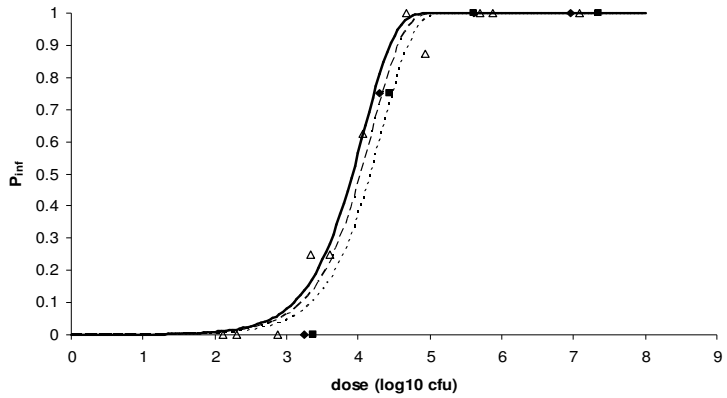

Figure 4.5. Dose response of* Salmonella *Typhimurium DT104 strains 2945 (■) and 911 (♦) cultured at pH 7 for positive WU rats six days after oral exposure to different doses and fitted dose-response models. *(…) 2945 exponential model, (--) 911 pH exponential model. Compared to the data (Δ) and the fitted exponential model (—) of the first experiment.*

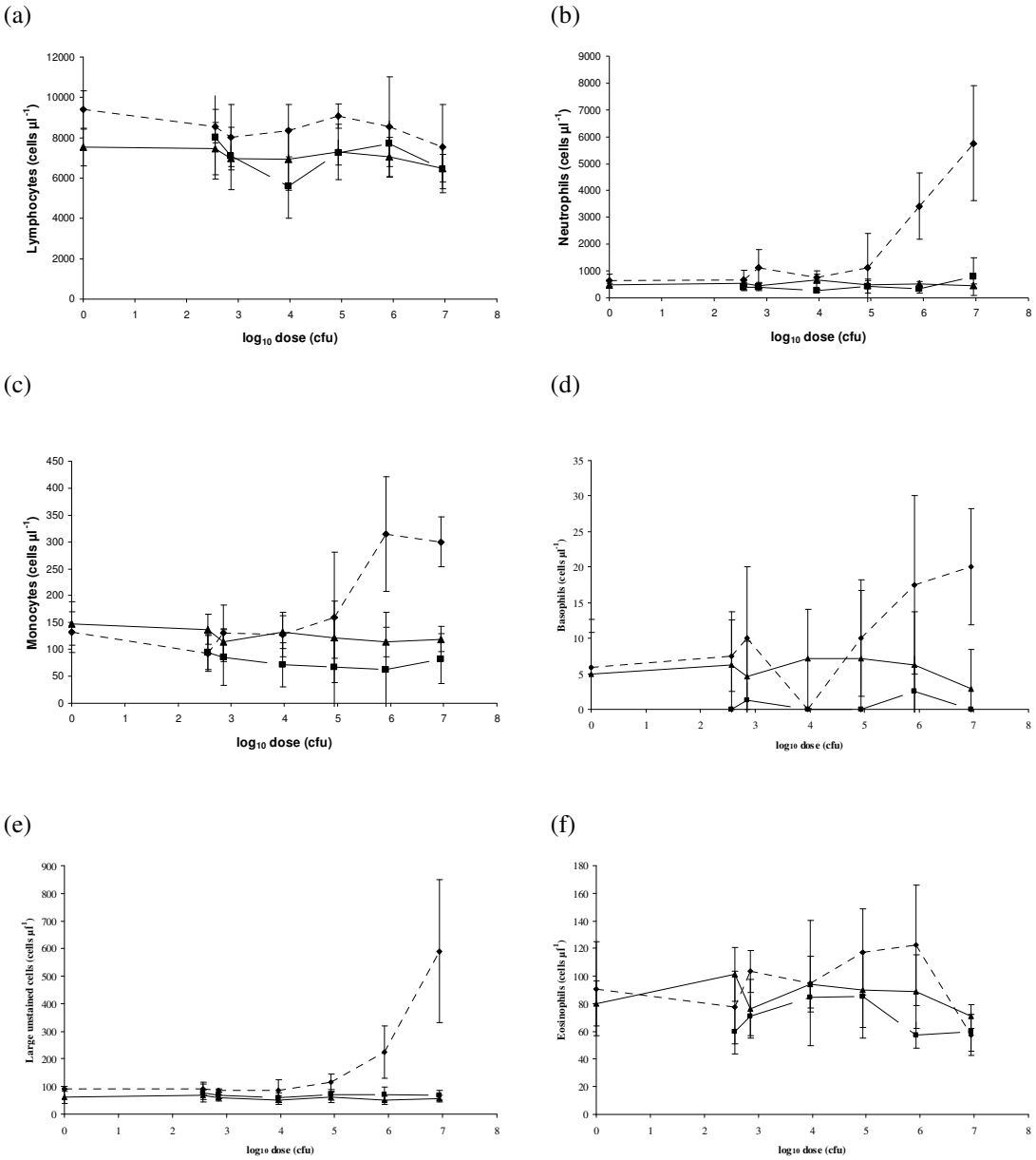

Figure 4.4. Dose-response relationship of white blood-cell subclasses counts in adult, male WU rats before and after exposure to* Salmonella *Typhimurium DT104 ATCC strain BAA-188. *Before exposure (▲), two days after exposure (■), six days after exposure (♦). Log dose 0 is the negative control group infected with* E. coli WG5 *(dose 5.62*10^6). (a) lymphocytes, (b) neutrophils, (c) monocytes, (d) basophils, (e) large unstained cells, (f) eosinophils*

IMPACT OF GROWTH PH AND STOMACH PASSAGE

It is known that the pH at which cells are grown may affect various cellular characteristics including virulence [23]. To assess the impact of growth pH on DT104 virulence, strains 911 and 2945 were cultured at pH 5, inducing acid resistance, and their virulence determined. The exponential model could be fitted to the data for strain 2945 (r = 1.37 x 10^{-4}, $ID50$=5.06*10^3, Fig. 4.6). For strain 911 cultured at pH 5 the Beta-Poisson model (α = 0.240, β = 275, r^2=0.98) fitted the data for infection better than the exponential model (r = 1.11 x 10^{-4}, r^2=0.67). The ID_{50} calculated with both models is approximately the same however (6.24 x 10^3 cfu for the exponential models and 4.67 x 10^3 cfu for the Beta-Poisson model, see Fig. 4.6). Also in this experiment similar changes in white blood cell population were found as in the first experiment (data not shown).

Culturing under acid adaptive conditions (at pH 5) can also result in improved survival of low pH environments (such as the stomach), as acid resistance is induced. The effect on stomach passage was further studied by assessment of DT104 strain virulence in rats without neutralizing the stomach with bicarbonate. Both strains 2945 and 911 were grown under acid-adaptive conditions (pH 5). The estimated dose-response parameters were r = 7.48 x 10^{-5} for 2945 and r = 2.42 x 10^{-5} for 911). This results in an ID_{50} of 9.26 x 10^3 cfu for strain 2945 and 2.87 x 10^4 cfu for strain 911 (Fig. 4.7). A similar change in white blood cell population was found in this experiment as in the previous three experiments.

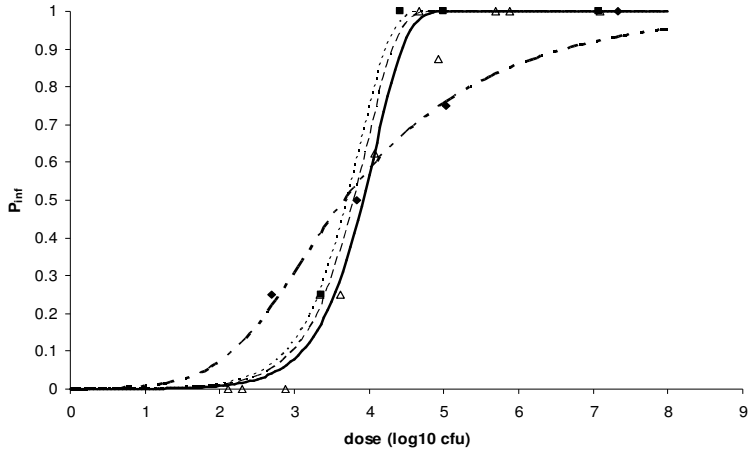

Figure 4.6. Dose response of* Salmonella *Typhimurium DT104 strains 2945 (■) and 911 (♦) cultured at pH 5 for positive WU rats six days after oral exposure to different doses and fitted dose-response models. *(…) 2945 exponential model, (--) 911 pH exponential model, (— – —) 911 pH 5 Beta-Poisson model. Compared to the data (Δ) and the fitted exponential model (—) of the first experiment.*

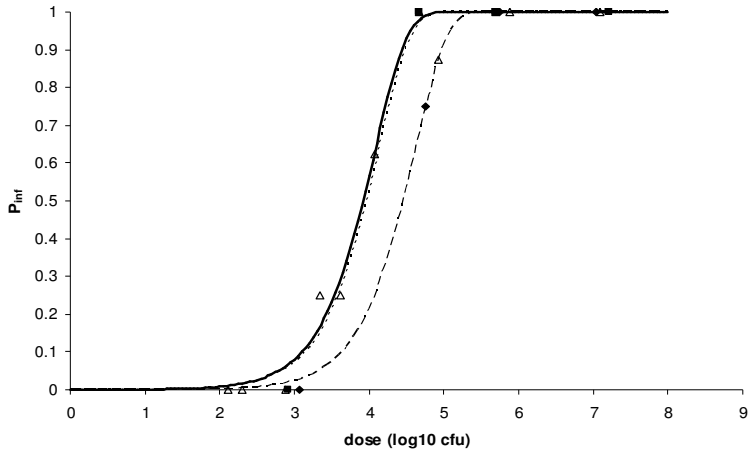

Figure 4.7. Dose response of* Salmonella *Typhimurium DT104 strains 2945 (■) and 911 (♦) cultured at pH 5 and administered without bicarbonate for positive WU rats six days after oral exposure to different doses and fitted dose-response models. *(…) 2945 exponential model, (--) 911 pH exponential model. Compared to the data (Δ) and the fitted exponential model (—) of the first experiment.*

IMPACT OF GROWTH PHASE

The ability of *Salmonella* to enter mammalian cells *in vitro* was shown to be affected by different phases of growth[24;25]. In this experiment bacterial cultures were harvested in the exponential phase. Strain 2945 and 911 were grown in LBG pH 7 to an OD_{660} of approximately 0.2, where-after the cells were harvested and prepared for oral admission. The fitted exponential model parameter for strain 911 was $r = 1.79 \times 10^{-3}$ (Fig. 4.8). From this the ID_{50} was calculated as 3.88×10^{2} cfu. For strain 2945 no model could be fitted because even at the lowest dose all animals were positive at day 6, but since the lowest dose inoculated was 2.50×10^{2} cfu ml^{-1} the ID_{50} is less than 2.50×10^{2} cfu. Therefore we examined also the data at day 2 (Fig. 4.9). The exponential model could be fitted to these data ($r = 1.15 \times 10^{-3}$ for 2945 and $r = 3.71 \times 10^{-4}$ for 911). The calculated ID_{50} was 6.02×10^{2} cfu for strain 2945 and 1.87×10^{3} cfu for strain 911. Notably, on day six in the high dose groups the bacterial counts in the spleen of the animals were much higher in the animals inoculated with the exponential phase cultures than in animals inoculated with stationary phase cells (Table 4.1). For the exponential phase cultures also the changes in white blood cell population were already seen on day 2 (data not shown) while for others this increase was seen only on day 6 (Fig. 4.3 and 4.4). In Figure 4.10 an overview is given of all calculated ID_{50}'s on day 6.

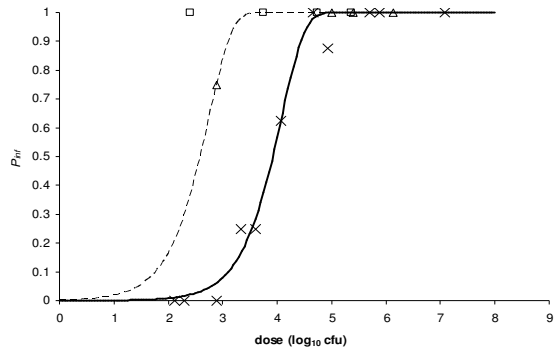

Figure 4.8. Dose response of* Salmonella *Typhimurium DT104 strains 2945 (□) and 911 (Δ) cultured at pH 7 to the exponential phase for positive WU rats six days after oral exposure to different doses and fitted dose-response models. *(--) 911 pH exponential model, compared to the data (x) and the fitted exponential model (—) of the first experiment. No model could be fitted for strain 2945 since all animals were positive at the lowest dose.*

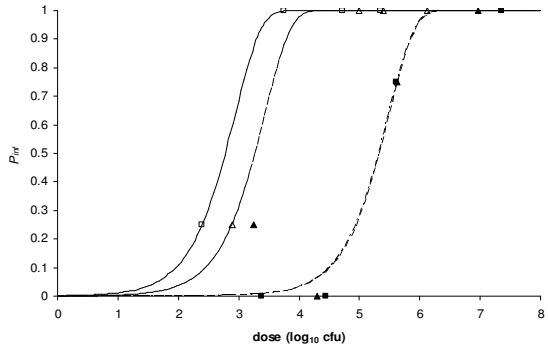

Figure 4.9. Dose response of* Salmonella *Typhimurium DT104 strains 2945 (■□) and 911 (▲Δ) cultured to the stationary (■▲) or the exponential (□Δ) phase for positive WU rats two days after oral exposure to different doses and fitted dose-response models. *(— —) 2945 stationary phase exponential model, (—) 2945 exponential phase exponential model, (— – —) 911 stationary phase exponential model, (--) 911 exponential phase exponential model.*

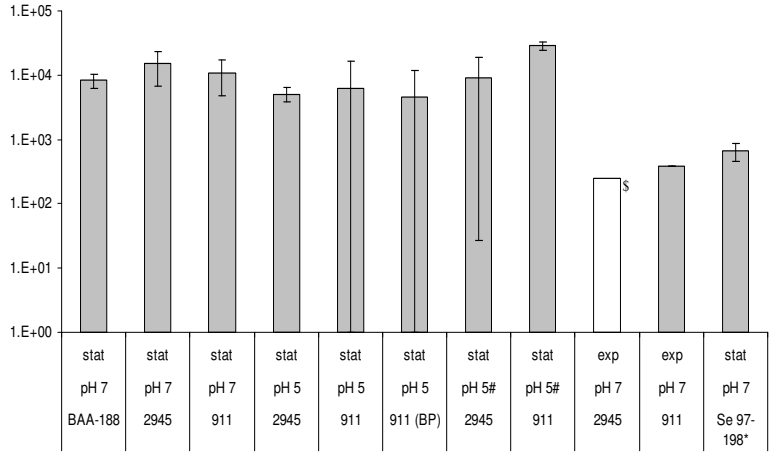

Figure 4.10. Calculated ID_{50} with 95% confidential level, six days after infection from all strains and culture conditions (stat = stationary growth phase; exp = exponential growth phase). *For all experiments the ID_{50} was calculated using the exponential model, for strains 911 cultured at pH 5 also the ID_{50} calculated by using the Beta-Poisson (BP) model is shown.*
$^{\$}$No ID_{50} could be calculated since all animal were positive at the lowest dose (2.50 x 10^2);
$^{\#}$Administered without bicarbonate;
** Adapted from [22]*

Table 4.1. Colony forming units in the spleen six days after inoculation with different doses of the S. Typhimurium DT104 strains

	2945				911			
dose group (log cfu)	pH 7 stat	pH 5 stat	pH 5 stat *	pH 7 exp	pH 7 stat	pH 5 stat	pH 5 stat*	pH 7 exp
2.5 – 3.5	-	3.65	-	4.10	-	2.90	-	4.04
3.5 - 5	2.89	3.59	3.67	4.19	3.14	3.84	3.79	4.19
5 - 6	3.63	3.68	3.52	**6.18**	3.59	3.68	3.67	3.74
6 – 7.5	3.60	3.49	3.66	**6.19**	3.44	3.78	3.70	**5.68**

All cultures are grown to the stationary phase and administered with bicarbonate unless indicated differently (* without bicarbonate) (stat is grown to the stationary phase, exp is grown to the exponential phase). Each data point indicates the log_{10} mean cfu in the spleen of positive animals. Bold figures indicate a higher count of S. Typhimurium DT104 in the spleen than with other growth conditions or lower doses. - no infected animals.

DISCUSSION

The dose response relationship of exposure by intragastric gavage of adult, male WU rats to *S.* Typhimurium DT104 showed to be a reproducible model, as was also found before with *S.* Enteritidis [22]. In all experiments after six days *Salmonella* was found in the ileum, Peyers patches and the spleen from approximately dose 10^3 onwards. No *Salmonella* was found in the jejunum, except for animals infected with bacterial cultures grown to the exponential phase. None of the animals developed a severe illness or diarrhea. In this study it is demonstrated that there is a clear dose-response relationship for the total white blood cell population on day 6. Takumi *et al.* [33] investigated neutrophil and monocyte response for *Salmonella* Enteritidis in a time dependent manner. In that study in the first phase (one day after infection) neutrophils and monocytes in the blood migrated to the infected tissue which resulted in a decrease of neutrophil and monocyte count in the blood. In the second phase, the bone marrow production exceeded the loss by migration and the neutrophil and monocyte count increased gradually in the blood with the maximum on day 4. In the third phase (from day 7) the number of neutrophils and monocytes released from the bone marrow decreased with the bacterial burden. In the current study the increase of white blood cells was found on day 6 (phase two of the time-dependent response) which is in agreement with the findings of Takumi *et al*. However in our sixth experiment using exponential phase bacteria the highest count of neutrophils and monocytes was found already on day 2 (phase two) and already decreased on day 6 (phase three). Therefore it can be concluded that the onset, the duration and the maximum of the three phases are dependent on the growth state of the strains.

In our study the ID_{50} s of *S.* Typhimurium DT104 grown to the exponential phase was 30-60 times lower than that of cells from the corresponding strains isolated at stationary phase. This implies that DT104 cultures grown to the exponential phase are clearly more infectious than cultures grown to the stationary phase. Previous *in vitro* studies also revealed increased virulence of *Salmonella* exponential phase cells i.e. their invasion capacity was higher than that of stationary phase cells [24;25] . Increased virulence during exponential growth may be explained by the observation that genes involved in the adhesion process are maximally expressed during this growth phase. Hermans *et al.*[34] found in microarray studies using *S.* Typhimurium DT104 grown to different growth phases, that genes of the *Salmonella* Pathogenicity Island 1 (SPI-1) were upregulated at the end of the exponential phase. Several SPI-1 effector proteins are known to be involved in the invasion process of *Salmonella* (reviewed in [35]), therefore upregulation of SPI-1 genes can result in increased invasion and thus increased virulence. In addition, Hermans *et al.*[34] have shown that LPS genes, which are thought to be important in the adhesion process, are also upregulated during exponential growth. Torres *et al.* [36] showed that the *lpf* (long polar fimbria) genes are maximally expressed during the mid exponential phase in

Enterohemorrhagic *Escherichia coli*. In *Salmonella* the long polar fimbriae are found to mediate adhesion to M cells[37] and the cells of the Peyers patches of the small intestine in a mouse model of infection [38]. They are therefore important at an early step of disease by an oral route of infection. It is conceivable that expression of *lpf* genes in *Salmonella* displays similar growth phase-dependent expression as in *E. coli*.
Growth at pH 5 induces acid resistance. This could lead to higher levels of infection as acid resistant strains can survive the acidic barrier of the stomach. However, i*n vivo* no effect of growth pH on the level of infection was found. Previously, in *in vitro* studies in Caco-2 and IEC-18 cells, also no differences were found in adhesive and invasive capacity, as well as in the induction of an immune response by *S.* Typhimurium DT104 cells grown at either pH 5 or pH 7 (unpublished results). No differences in *S.* Typhimurium DT104 virulence were found when oral doses were administered with or without bicarbonate to neutralise the gastric acid. This is probably due to the fact that the pH of the rat stomach is higher than that of the human stomach, pH 3.5 for rats *versus* pH 2 for humans [39;40]. Notably, all *S.* Typhimurium DT104 previously tested for low pH tolerance, were able to survive after 2 hours at pH 3.5 (unpublished results). It is conceivable that in the rat model used here, all administered *Salmonella* will reach the intestine since no inactivation will occur in the stomach. The model used in this study is therefore very useful for investigating the effects of strains diversity, growth conditions and food matrices at the site of infection i.e. the small intestine, but does not allow for assessment of the efficiency of human gastric passage. The ID_{50} from the *S.* Typhimurium DT104 strains used in this study are higher than of a *S.* Enteritidis strain used in the same rat model by Havelaar *et al* [22].

The results obtained in this study will be used to determine the relation between *in vitro* and *in vivo* virulence data, and this will subsequently be used to predict the DT104 virulence in humans using a parallelogram approach. In this study it was shown that the physiological state of the bacteria can be of great importance for the *in vivo* virulence. Therefore when studying dose response relationships one has to take into account in which state the bacteria are during the experiment. Furthermore it is important to standardise the method used since large differences can be found. *Salmonella* is a causative agent of food borne disease. Also when setting food safety objectives it is important to consider that *Salmonella* are more virulent in the exponential than in the stationary phase. Since it is difficult to determine whether bacteria present in food are in the exponential or stationary phase, a worst case scenario has to be taken into account.

ACKNOWLEDGEMENTS

We thank Hans Strootman, Piet van Schaik, Dirk Elbers, Christine Soputan and Coen Moolenbeek for the realisation of the animal experiments, Liset de la Fonteyne and Yvonne Wallbrink for performing the haematological analysis and Ellen Delfgou, John Dufrenne, Michal Voorbergen, Luc Wijnands and Frans van Leusden for assisting with the microbiological analysis. We also thank the Nutrition, Health, Safety and Sustainability program of the Netherlands Organisation for Health Research and Development for funding this research.

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CHAPTER 5

Prediction of the probability of infection of *Salmonella Typhimurium* DT104 in humans using a parallelogram approach.

P.A. Berk, M.H. Zwietering and R. de Jonge

ABSTRACT

The risk of infection with a pathogenic organism can be estimated following different approaches. In this study for the first time a parallelogram approach is used to predict the probability of infection for humans of *Salmonella* Typhimurium DT104 in the intestines combining both *in vitro* and *in vivo* data. The level of invasion in cell lines seems to give a good prediction on the infectivity of a *Salmonella* strain, since the level of invasion in the rat cell line is correlated with the infectivity in rat. Therefore these two parameters are used in the parallelogram approach. As a result of this parallelogram approach the log dose at which 50% of the humans is infected (ID_{50}) varied from 2.86 – 5.33. Acid resistance was combined with the parallelogram approach to predict the probability of infection in humans (P_{inf}), since stomach survival is also an important virulence factor for food borne bacteria. *Salmonella* grown to the exponential phase have a 30-60 times higher probability of causing an infection than grown to the stationary phase. However acid resistant strains have a 100 000 times higher probability of causing an infection than acid sensitive strains. In combination with acid resistance (stomach survival), the parallelogram approach can give a good prediction of the infective dose of (new emerging) infective strains of *Salmonella* in human. Secondly it can be concluded that growth phase and strain are important virulence determinants, but that acid resistance is the main determinant of virulence for *Salmonella.*

INTRODUCTION

Infections caused by *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) and Typhimurium (*S.* Typhimurium) continue to be a major health problem. They usually occur after consumption of contaminated food or water and can manifest in a variety of disease states, ranging from asymptomatic carrier status through gastroenteritis or even severe systemic infection that can occasionally even lead to death. Among the different *Salmonella* strains the nature and severity of the infection varies. Because of the severity of disease caused by multi antibiotic resistant *S.* Typhimurium phage type DT104 (*S.* Typhimurium DT104) and the increasing frequency of isolation from patients of this *Salmonella* strain, *S.* Typhimurium DT104 has been proposed to be more virulent than other *S.* Typhimurium strains.

The clinical course of human salmonellosis is usually characterized by fever, abdominal pain, diarrhoea, nausea and sometimes vomiting. These symptoms are caused by the destruction by *Salmonella* of the intestinal epithelium. One important step in the cause of this destruction is invasion of *Salmonella* into intestinal cells. *In vitro*, a type III secretion apparatus encoded on *Salmonella* Pathogenity Island 1 (SPI-1) is required for invasion of mammalian cells [1]. Invasion of *Salmonella* is thought to be a crucial step in the pathogenesis, since bacteria with mutations in SPI-1 are attenuated in mice and calves [2;3]. M cells have often been suggested as the primary invasion site for *Salmonella spp* [4;5]. It is clear however that *Salmonella* can also reach the intestinal barrier through the epithelial cells (enterocytes) of the small intestine [6], and it has been argued that perhaps enterocytes may indeed constitute the main port of *Salmonella* entry since they vastly outnumber M cells [7]. The human Caco-2 cell line is an epithelial cell line known to spontaneously differentiate to form a polarized monolayer [8]. Although the cells are isolated from an adult human colon, they express several markers characteristic of normal small intestinal villus cells [9]. The Caco-2 cell line has been shown to be a good *in vitro* system for the analysis of *Salmonella* virulence characteristics [6;10;11]. In the early nineties filter-grown rat IEC-18 cells were used as a model to study small intestinal epithelial permeability. It was found that filter-grown IEC-18 developed a TER (approximately 20 to 45 $\Omega*cm^2$) comparable to that of the small intestine [12].

Experimental *Salmonella* infections are extensively studied in rodents. *In vivo* rats provide a useful model for some aspects of salmonellosis in humans, as intestinal transport and electrolyte transport patterns, important factors in *Salmonella* infections, are similar in rats and in humans [13].

Whereas in humans generally self-limiting gastroenteritis is observed, in rats and mice non-typhoid *Salmonellae* induce a systemic disease. Within 2 hours after oral exposure of rats to *Salmonella*, the bacteria can be detected in the distal ileum and caecum. Invasion occurs via

the M-cells in Peyers patches and the *Salmonella* can be detected in the mesenteric lymph nodes within 8 hours after infection [14] and from the mesenteric lymph nodes *Salmonella* can spread throughout the body. Both rat and human are sensitive for *Salmonella.* Although disease following infection in both rat and human are different, the rodent is particularly useful for investigating the initial stages of the pathological pathway. These initial stages consist of the invasion of the epithelium and the innate immune response. Havelaar *et al.* [15] showed that the male WU rat provides a useful model for studying these initial stages in rat. After oral inoculation the amount of *Salmonella* in the spleen provides a dose dependent level of infection. From this dose response model a probability of infection for rat was calculated by Havelaar *et al*.

The primary source of dose-response data has been human volunteer feeding studies. Such trials provide the most direct measure of human response to pathogens, which is its big advantage. However, these data do have limitations. Volunteers for these studies have been almost exclusively healthy adult males. Information on the susceptibility of higher risk subpopulations or potential gender effects is generally not available. Furthermore, volunteer studies are limited to few subjects and high doses.
In vitro (human) cell lines can be used to study specific steps of the pathogencitity pathway. This is a simple, relatively cheap system with all the variables under control, but its simplicity is also its disadvantage since processes are missing on the interaction between different cells, organs and the immune system. *In vivo*, animal models can be used to study the infectivity. The advantage of using animal models compared to cell lines is the fact that a whole organism can be studied. However it is important to select an appropriate animal model, in which the infection that is caused by the pathogen is according to the same mechanism of pathogenicity in both man and animal. The translation of both *in vitro* and *in vivo* model systems to humans is often difficult.

In toxicological studies a parallelogram approach is often used to study the effects of toxic substances. The parallelogram approach to extrapolate data to man was proposed in the late 1970's by Sobels [16]. It was originally used for extrapolation of chemical mutagenesis data. In this approach (see fig. 1) data derived from different *in vitro* cell systems can be compared with each other (A) and data from experiments with animal cells can be compared with *in vivo* animal data (B). Extrapolation can now follow two routes: from *in vivo* animal data to man (C) and from *in vitro* human data to man (D). In this study the risk of infection in man is predicted, for the first time, with the use of a parallelogram approach.

The probability of infections with food borne pathogens is also dependent on the ingested dose and the percentage of stomach survival of the bacteria. As the barrier function of the

rat and human stomach differ, stomach survival was also included in the prediction of the probability of infection.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

One *Salmonella* Enteritidis strain and five *Salmonella* Typhimurium DT104 strains were used in this study of which two are acid sensitive, two acid resistant and one intermediate (Table 5.1) [17].

Table 5.1 Strains used in this study

strain	*AR/AS*	*Isolation source*	*Reference*
327	AS	Food	1
911	AS	Human	1
2945	AR	Human	1
3633	AR	Food	1
BAA-188	Intermediate	Human	1
Se 97-198	AR	Livestock	2

AR = acid resistant, AS = acid sensitive, 1. [17]*, 2.* [15]*, Se =* Salmonella enterica *serovar Enteritidis*

All strains were stored at -70°C in 50% Brain Heart Infusion (BHI, Difco) broth plus 50% glycerol. Strains were allowed to recover from –70°C for 8 hours at 37°C in BHI without shaking, after which the bacteria were used in the different experiments. Growth media used in the different experiments consisted of LB broth (10 g l^{-1} NaCl, 10 g l^{-1} trypton (Oxoid) and 5 g l^{-1} yeast extract (Oxoid)) + 4 g l^{-1} glucose (LBG), buffered with 100 mmol l^{-1} 4-morpholine-propanesulfanic acid (Mops; Roche) at pH 7 or 100 mmol l^{-1} 2-morpholine-ethanesulfanic acid monohydrate (Mes; Merck) at pH 5. Cultures were grown in closed bottles without shaking. The different growth conditions used in both the *in vitro* and *in vivo* experiments are shown in table 5.2.

Table 5.2 Log invasion in Caco-2 cells, log invasion in IEC-18 cells and log r_{rat} for different strains and growth conditions.

Strain	Growth conditions	log invasion IEC-18	log invasion Caco-2	log r_{rat}
911	pH 7 stat	4.40	2.66	-4.20
	pH 5 stat	3.59	2.55	-4.62
	pH 7 exp	5.00	4.86	-2.75
2945	pH 7 stat	4.12	2.68	-4.33
	pH 5 stat	4.02	2.99	-4.62
	pH 7 exp	4.74	4.62	-2.56
BAA	pH 7 stat	3.56	2.29	-4.08
Se 97-198	pH 7 stat	4.83	4.04	-2.98

Stat = grown to the stationary phase, exp = grown to the exponential phase, r_{rat} = probability of one Salmonella causing an infection in a rat. Log invasion in both IEC-18 cells and Caco-2 cells was determined 2 hours after infection of the cell lines with 10^9 bacteria in a 12-wells plate.

IN VITRO INVASION DATA

In vitro data from two intestinal cell lines are used, the human Caco-2 cell line (see also Chapter 3) and the rat IEC-18 cell line. Passages 25-45 from Caco-2 cells and IEC-18 cells, obtained from the American Type Culture Collection (ATCC), were used in these experiments. Cells were grown confluently at 37°C and 5% CO_2 in air, in Dulbecco's modified Eagle's medium (DMEM) with 25 mM Hepes and 4.5 g l^{-1} glucose without sodium pyruvate (GibcoBRL, Life Technologies ltd, Paisley, Scotland). Fifty ml heat inactivated (30 minutes at 60°C) fetal bovine serum (Integro b.v., Zaandam, the Netherlands), 5 ml MEM non-essential amino acids (Gibco), 5 ml L-glutamine (final concentration 6 mmol l^{-1}; Gibco) and 0.5 ml Gentamicin (final concentration 0.5 µg ml^{-1}; Gibco) was added to 500 ml DMEM. For each experiment, cells were seeded at 160.000 cells per well in 12 well tissue culture plates (surface area per well is 401 mm^2)(Costar, Corning Costar Europe, Badhoevedorp, the Netherlands). Cells were grown confluently and used 12-19 days after plating; medium was changed 3 times a week. One hour before infection, medium was replaced by culture medium without serum and gentamicin, this is called tissue culture medium (TCM). *Salmonella* strains were cultured at both pH 7 and pH 5. Exponentially and stationary phase cultures of *Salmonella* (18 h, 37°C) were spun 10 minutes at 4500 x *g*. The pellets were concentrated in TCM to 2.5 x 10^{10} cfu ml^{-1} to obtain equal concentrations. Each well with intestinal cells was inoculated with 40 µl bacterial suspension. Two hours after infection, cells were washed 3 times with TCM and incubated an additional 3 hours with 1 ml 300 µg ml^{-1} gentamicin and 300 µg ml^{-1} ciprofloxacin in

TCM. After incubation cells were washed 3 times with TCM and lysed with 1 ml 1% Triton-X100. Invasion was determined by counting colony-forming units (cfu), after serial dilution in peptone buffered physiological salt (pps), on trypton soya agar (TSA)-plates. Plates were counted after 18 hours incubation at 37°C. All tests were performed five times.

IN VIVO DATA FOR RAT

Salmonella-free male Wistar-Unilever rats, obtained from the breeding colony at the National Institute of Public Health and the Environment, Bilthoven, the Netherlands, were infected with different doses of the respective *Salmonella* strain. The exponential or Beta Poisson model was fitted to the data. From these models an infection dose at which 50% (ID_{50}) of the animals were infected was calculated [18].

DOSE-RESPONSE MODELLING OF IN VIVO DATA.

Dose-response modelling was based on the following assumptions: (1) single hit, i.e. one single surviving organism is capable of initiating an infection[19]; (2) independent action, i.e. the probability of one organism initiating infection is independent of the presence of other organisms[20]; and (3) random distribution of the organisms in the inoculum[21]. If it is assumed that the probability of any organism in any host to survive and initiate infection has a constant value r, then the exponential model follows:

$$P_{inf} = 1 - e^{-rD} \qquad \text{Equation 1}$$

where P_{inf} is the probability of infection and D is the mean dose entering the intestine and r is the probability of one *Salmonella* to infect the host. The D can be calculated from the initial dose ingested and the percentage of bacteria surviving the stomach.
The initial dose on different food products is variable and often not known with high accuracy (only the prevalence is often determined and reported). Therefore different scenarios are evaluated.
The percentage of bacteria that survives the stomach is dependent on the acid resistance of the strain, but also in certain cases on the food product and on host parameters (e.g. age). In this study of these factors only the effects of acid resistance of the strains is taken into account.

THE PARALLELOGRAM APPROACH

In the first step of the parallelogram approach the relations between the different data are described. In figure 5.1 each arrow represents a relationship which can be described by an equation:

Route 1:
Data obtained in *in vitro* studies using a rat cell line can be compared to data obtained *in vitro* using a human cell line (arrow A). Assuming that the relationship between rat cell line and human cell line parallels with the relation between rat and human *in vivo*, this relationship can then be used to calculate human *in vivo* data from *in vivo* data obtained by experiments using rat (arrow C).

Route 2:
Data obtained in *in vitro* studies using a rat cell line can be compared to *in vivo* data obtained with rat experiments (arrow B). Assuming that the relationship between a rat cell line *in vitro* and a rat parallels with the relation between a human cell line and humans, this relationship can then be used to calculate *in vivo* data for humans from *in vitro* experiments using a human cell line (arrow D).

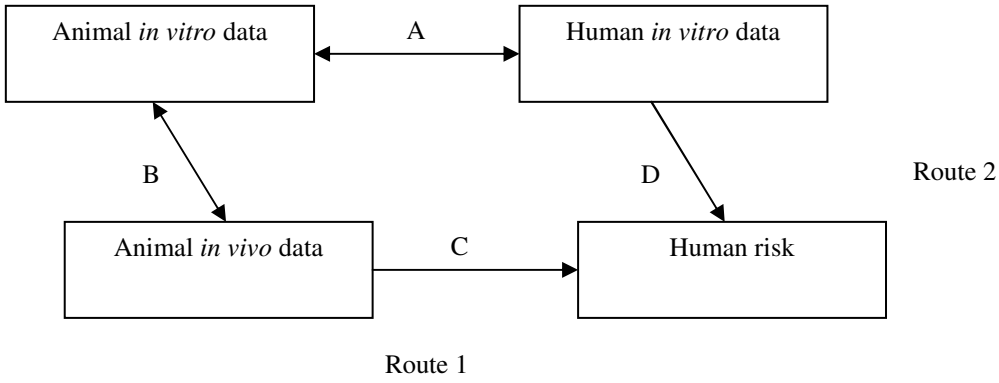

Figure 5.1 The parallelogram approach first described by Sobels [16]. *Data derived from different in vitro cell systems can be compared with each other (A) and data derived from experiments with animal cells can be compared with in vivo animal data (B). Extrapolation can now follow two routes: from in vivo animal data to man (assuming A = C, route 1) and from in vitro human data to man (assuming B = D, route 2).*

RESULTS

PARALLELOGRAM APPROACH

To determine the effect of growth pH and growth phase in *in vivo* studies, using a rat model, strains 911 and 2945 were cultured at both pH 7 and pH 5 to the stationary phase and at pH 7 also to the exponential phase. Therefore the same conditions used in the *in vivo* studies were used to culture *Salmonella* prior to exposure to the Caco-2 cell line and the IEC-18 cell line to make a comparison between *in vitro* and *in vivo* results. The conditions and the invasive capacity in Caco-2 cells and IEC-18 cells as well as the probability of infection for one bacterium in rat (r_{rat}) is shown in table 5.2.

To calculate the probability of one bacterium causing an infection in humans (r_{human}), two routes can be followed in the parallelogram approach and for both routes two scenario's are taken into account.

Route 1. *In vitro* parallel to *in vivo*.
Correlation and regression analysis were used to determine a linear correlation between the invasion *in vitro* in the IEC-18 cell line with the invasion *in vitro* in the Caco-2 cell line and to describe this correlation.

Scenario A: Different strains and growth conditions give a proportional effect on both the invasion of Caco-2 cells and on the invasion of IEC-18 cells. For example when the invasion in Caco-2 cells is x times higher due to a certain condition than the invasion in IEC-18 cells will also be x times higher. On a linear scale this can be translated as follows: y = ax, translated to a logarithmic scale this will be for route 1:

$$\log \text{invasion}_{\text{Caco-2}} = \log \text{invasion}_{\text{IEC-18}} + \log a \qquad \text{Equation 2}$$

Assuming that this same proportionality also holds for the translation from rat *in vivo* to human *in vivo* we get:

$$\log r_{\text{human}} = \log r_{\text{rat}} + \log a \qquad \text{Equation 3}$$

The correlation coefficient (r^2) between the log invasion in Caco-2 cells and the log invasion in IEC-18 cells was 0.67 and the estimation for parameter log a is -0.9468 (95% CI -1.434 – -0.460)

Scenario B: When the effect of different strains and growth conditions is not a proportional effect, the effect can follow a power law. On a linear scale this can be translated as: $y = bx^c$, translated to a logarithmic scale this will be, for route 1:

$$\log \text{invasion}_{\text{Caco-2}} = c\ (\log \text{invasion}_{\text{IEC-18}}) + \log b \qquad \text{Equation 4}$$

Analogous:

$$\log r_{\text{human}} = c\ (\log r_{\text{rat}}) + \log b \qquad \text{Equation 5}$$

The correlation coefficient (r^2) between the log invasion in Caco-2 cells and the log invasion in IEC-18 cells was 0.79. The estimated parameters for this equation are; log b = -3.6615 (95% CI -7.2904 – -0.0326) and c = 1.6338 (95% CI 0.7926 – 2.4750)

In figure 5.2 the regression line of both scenarios is shown. The probability of infection of one bacterium causing an infection in humans (r_{human}) is calculated for both scenarios and shown in table 5.3.

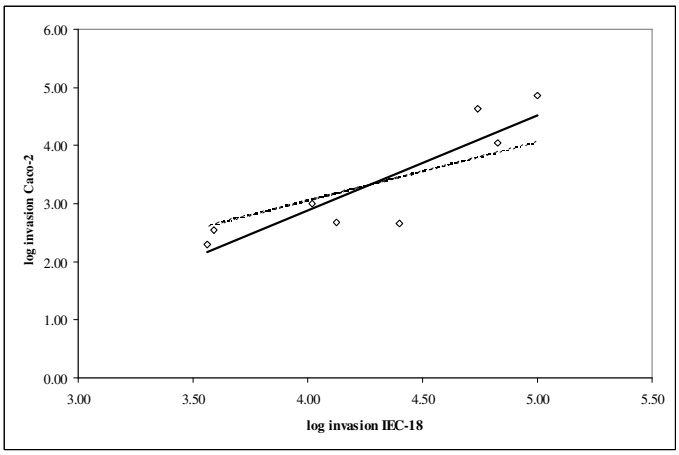

Figure 5.2 Regression lines between log invasion in Caco-2 cells and IEC-18 cells. *Different data points represent different S. Typhimurium DT104 strains under a certain growth condition and one* S. *Enteritidis strain. Two scenario's are taken into account; A) Different strains and growth conditions give a proportial effect on both Caco-2 and IEC-18 cells (interrupted line) and B) different strains and growth conditions follows a power law (continuous line).*

Table 5.3 Calculated r_{human} using route 1 of the parallelogram approach

Strain	*Growth conditions*	*Log r_{rat}*	*Calculated log r_{human}*	
			A	B
911	**pH 7 stat**	-4.20	-5.28	-10.74
	pH 5 stat	-4.62	-5.07	-10.40
	pH 7 exp	-2.75	-3.50	-7.84
2945	**pH 7 stat**	-4.33	-5.14	-10.52
	pH 5 stat	-4.13	-5.56	-11.20
	pH 7 exp	-2.56	-3.69	-8.15
BAA	**pH 7 stat**	-4.08	-5.02	-10.32
Se 97-198	**pH 7 stat**	-2.98	-3.92	-8.52

Calculated probability of one bacterium causing an infection in humans (log r_{human}) using route 1 of the parallelogram approach. Scenario A: assuming different strains and growth conditions give a proportial effect on both Caco-2 and IEC-18 cells. Scenario B: assuming different strains and growth conditions follows a power law. stat = stationary phase, exp = exponential phase, Se = Salmonella *Enteritidis.*

Route 2. Rat parallel to human.

Correlation and regression analysis were used to determine a linear correlation coefficient between the invasion *in vitro* in the IEC-18 cell line with the *r* in the rat (r_{rat}) and to describe this correlation.

Scenario A: Effect of different strains and growth conditions gives a proportional effect on both the invasion of IEC-18 cells and on the probability of infection in rat. For example when the invasion in IEC-18 cells is x times higher due to a certain condition than probability of infection in rat will also be x times higher. On a linear scale this can be translated as follows: y = dx, translated to a logarithmic scale this will be for route 2:

$$\log r_{rat} = \log \text{invasion}_{IEC\text{-}18} + \log d \qquad \text{Equation 6}$$

Analogous:

$$\log r_{human} = \log \text{invasion}_{Caco\text{-}2} + \log d \qquad \text{Equation 7}$$

The correlation coefficient (r^2) between the log probability of one bacterium causing an

infection in rat and the log invasion in IEC-18 cells was 0.67 and the estimation for parameter log d is -7.9870 (95% CI -8.357 – -7.617).

Scenario B: When the effect of different strains and growth conditions is not a proportional effect, the effect will follow a power law. On a linear scale this can be translated as: $y = ex^f$, translated to logarithmic scale this will be, for route 2:

$$\log r_{rat} = f\ (\log invasion_{IEC\text{-}18}) + \log e \qquad \text{Equation 8}$$

Analogous:

$$\log r_{human} = f\ (\log invasion_{Caco\text{-}2}) + \log e \qquad \text{Equation 9}$$

The correlation coefficient (r^2) between the log probability of one bacterium causing an infection in rat and the log invasion in IEC-18 cells was 0.67. The estimated parameters for this equation are; log e = -9.0568 (95% CI -12.339 – -5.775) and f = 1.2498 (95% CI 0.489 – 2.011).

In figure 5.3 the regression line of both scenarios is shown. The probability of infection of one bacterium causing an infection in humans (r_{human}) is calculated for both scenarios and shown in table 5.4.

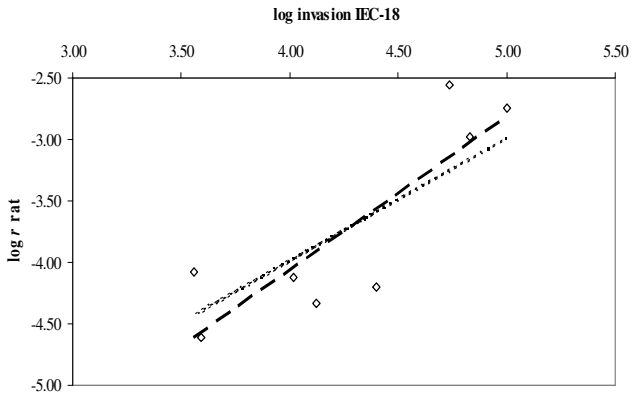

Figure 5.3 Regression lines between log invasion in IEC-18 cells and the log r in rat. *Different data points represent different S. Typhimurium DT104 strains under a certain growth condition and one* S. *Enteritidis strain. Two scenario's are taken into account; A) Different strains and growth conditions give a proportial effect on both rats and IEC-18 cells (continuous line) and B) different strains and growth conditions follows a power law (interrupted line).*

Table 5.4 Calculated r_{human} using route 2 of the parallelogram approach

Strain	*Growth conditions*	*Log invasion$_{Caco-2}$*	*Calculated log r_{human}*	
			A	B
911	**pH 7 stat**	2.66	-5.30	-5.59
	pH 5 stat	2.55	-5.00	-5.19
	pH 7 exp	4.86	-3.36	-3.07
2945	**pH 7 stat**	2.68	-5.32	-5.61
	pH 5 stat	2.99	-5.44	-5.76
	pH 7 exp	4.62	-3.13	-2.76
BAA	**pH 7 stat**	2.29	-5.70	-6.09
Se 97-198	**pH 7 stat**	4.04	-3.95	-3.83

Calculated probability of one bacterium causing an infection in humans (log r_{human}) using route 2 of the parallelogram approach. Scenario A: assuming different strains and growth conditions give a proportial effect on both rats and IEC-18 cells. Scenario B: assuming different strains and growth conditions follows a power laws. stat = stationary phase, exp = exponential phase, Se = Salmonella *Enteritidis..*

To determine which scenario should be used to calculate the probability of infection of one bacterium causing an infection, the log *r* obtained using both scenario's from both routes is compared to the log *r* obtained by human feeding studies. In table 5.5 the results are shown from human feeding studies in the fifties[18-21].

Table 5.5 Log r obtained with human feeding studies in the fifties

***Salmonella* strain**	**Log *r***
S. Anatum I	-4.50
S. Anatum II	-4.36
S. Anatum III	-5.57
S. Meleagridis I	-4.29
S. Meleagridis III	-5.66
S. Newport	-5.25
S. Bareilly	-4.85

Adapted from McCullough and Eisele 1951 (ref 23-26). The fraction infected individuals per dose was plotted against the dose. The log r was calculated using the exponential dose-response model.

Both scenario's from route 2 result in a calculated probability of infection of one bacterium causing an infection in humans comparable to that of the human feeding studies. However, for route 1 both scenario's result in different probabilities of one bacterium causing an infection in humans. The results obtained with scenario A are comparable to that of the human feeding studies and route 2.

Also statistically it is reasonable to believe that the effect on the different assays is a proportional effect. Both c (route 1) and f (route 2) from scenario B are statistically not different from 1, since for both regression lines 1 falls within the 95% confidence interval of the slope. When assuming that both c and f are 1, the equations of scenario A apply.

The results from scenario A from both routes are therefore used to calculate the probability of infection for humans (P_{inf}).

PROBABILITY OF INFECTION IN HUMAN

With the probability of one *Salmonella* causing an infection (r) the probability of infection for human can by calculated by using equation 1, where D is the dose reaching the intestine.
The probability of infection for humans ($P_{inf} = 1\text{-}e^{-rD}$) for strains 2945 and 911 grown at pH 7 to the stationary or exponential phase is shown in figure 5.4, where r_{human} is calculated using the parallelogram approach as described previously and D is varied from 1 to 10000, to determine the effect of dose on the probability of infection. Both strains behaved similar; however both *Salmonella* strains grown to the exponential phase have a probability of infection that is about 30-60 times higher than that of bacteria grown to the stationary phase.

The dose reaching the small intestine is dependent on both the ingested dose and the stomach survival. The rat model is not a representative model for human with regard to the stomach passage. The pH of the empty stomach of rat is approximately 3.5 whereas the pH of the empty human stomach is around 2 [22]. After consumption of a meal the pH raises. On average *Salmonella* remains in the stomach for two hours at pH 2.5. Stomach survival measurements after 2 hours at pH 2.5 can be performed under laboratory conditions in an acid resistant test [17].
For strain 2945 (acid resistant) approximately 36% of the bacteria survived after 2 hours at pH 2.5 and for strains 911 (acid sensitive) approximately 0.00027% of the bacteria survived after 2 hours at pH 2.5 [17]. The effect of acid resistance on the probability of infection at different ingested doses is shown in figure 5.5. Acid resistant strains have an almost 100 000 higher probability of infecting human than acid sensitive strains.

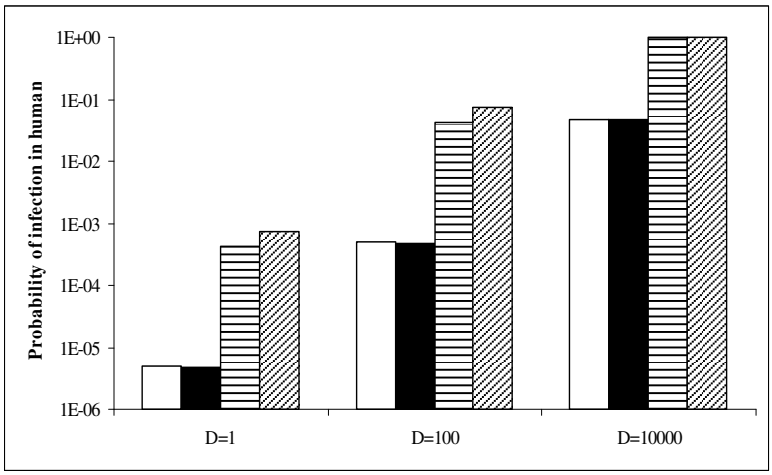

Figure 5.4 Effect of strain, growth phase and dose in the intestine on the probability of infection in human. *Open bars = strain 2945 grown to the stationary phase, Closed bars = strain 911 grown to the stationary phase, Horizontal striped bars = strain 2945 grown to the exponential phase, Cross striped bars = strain 911 grown to the exponential phase. D = infectious dose*

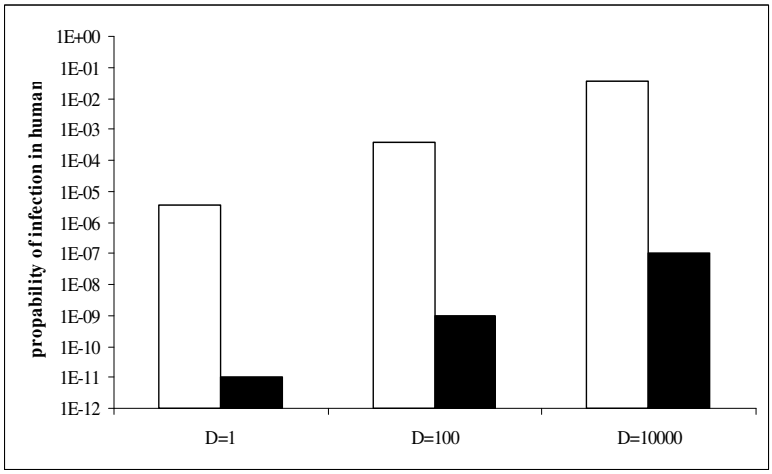

Figure 5.5 Effect of acid resistance on the probability of infection in humans. *Open bars = acid resistant strain 2945 (stomach survival 36%), closed bars = acid sensitive strain 911 (stomach survival 0.00027%). D = infectious dose*

DISCUSSION

In this study the parallelogram approach was used for the first time to predict a dose-response relationship of *Salmonella* in humans. From this study we can conclude that the invasive capacity *in vitro* of a certain *S.* Typhimurium DT104 strain culture can be a good representation for the infectivity of this strain *in vivo*. Using the parallelogram approach it was found that growth phase is an important virulence parameter for *Salmonella.*

Invasion by *Salmonella* is necessary for causing infection in humans and rats. Route 1 of the parallelogram approach signifies the correlation between invasion in rat and human epithelial intestinal cells, and represents the relation between the whole infection processes in rat and human. Therefore, in route 1 of the parallelogram approach a translation is made from *in vitro* cell lines to a whole organism *in vivo*. Using route 2 a translation is made from *in vitro* invasion in a cell line to an *in vivo* infection in a whole organism.

For both routes, two scenarios are taken into account. In the first scenario (A) it is assumed that the effect of strain differences and growth conditions gives a proportional effect on the invasion in either human or rat intestinal cell lines or the probability of one bacterium causing an infection in rats. In the second scenario (B) it is assumed that the effect of different strains and growth conditions follows a power law. Following route 2 both scenario's result in approximately the same values for the probability of one bacterium causing an infection in humans. Following route 1 both scenarios result in very different values for the probability of one bacterium causing an infection in humans. The results found using scenario A are comparable to that of route 2. This is plausible, since there is no reason to expect that a variation in growth pH of the organism, for example, has different effect on the infectivity in human cell-lines than in rat cell-lines. Also statistically it is likely to use scenario A to calculate the probability of one bacterium causing an infection in humans, since the more complex power law does not give a significant improvement compared to, the more simple, proportional approach.

Following scenario A from both route 1 and route 2; the log r_{human} varies from -5.56 to -3.00, which means that the log ID_{50} found for humans in this study varies from 2.84 – 5.40. This is comparable to ID_{50} found in human feeding studies in the fifties [23-26] and ID_{50} values calculated from *Salmonella* outbreak data [27]. In the human feeding studies the *Salmonella* was administered in a glass of eggnog shortly following a meal. Although passage through the stomach is present it is likely that a high percentage of this *Salmonellas* have survived this passage. This is caused by the fact that the *Salmonella* is administered shortly after a meal. Ingestion of a meal can immediately raise the pH to about 5 or 6 [28 29].

During this period, potentially disease-causing bacteria can be transported to the small intestine without being destroyed by low pH. Takumi *et al.* [30] showed, by modeling the gastric pH and transport, that this was indeed the case for *E. coli*. For healthy young adults 20-73% of the ingested bacteria could survive the stomach and arrive in the small intestine. The *r* value from scenario B from both routes is used to calculate the probability of infection for human.

The probability of infection is dependent on the *r* and *D*. The *D* is the amount of *Salmonella* reaching the small intestine and is dependent on the ingested dose and the survival in the stomach. The ingested dose is dependent on the contamination level of the food product. The level of *Salmonella* on a food product has a high variability. For *S.* Typhimurium in cheddar cheese D'Aoust *et al.* [31] found 1.5 to 9 cfu per gram, in ice cream 1130-11300 cfu per gram was found by Armstrong *et al.* [32] and $1*10^8 – 1*10^9$ cfu per gram was found by Taylor *et al.* [33]. In 1996 Hennesy *et al.* [34] found 0-6 cfu per gram of *S.* Enteritidis in ice cream and Levy *et al.* [35] found $1*10^4 – 1*10^5$ cfu per gram in hollandaise sauce.

In rat the dose reaching the intestine (*D*) is very likely to be equal to the ingested dose, since the stomach pH of the rat (3.5) is not lethal for *Salmonella*. In human however also the stomach survival of the *Salmonella* is important in determining the dose reaching the intestine (*D*). Under fasting conditions, the pH in healthy volunteers is around two [22], a pH value that *Salmonella* can survive, but only for a short period of time. In this study it was shown that an acid resistant strain has about 100 000 times higher probability of surviving this low pH and thus of causing an infection in human than acid sensitive strains.

From this study we can conclude that the *in vitro* invasive capacity of a certain *S.* Typhimurium DT104 strain culture can be used to predict its infectivity *in vivo*. If this finding is founded by additional data, it will make it possible to reduce the use of laboratory animals and in the future maybe even replace them. Secondly it can be concluded from this study that growth phase, but more importantly, acid resistance is the main virulence parameter for *Salmonella,* provided the organism is exposed to pH values as low as 2.5 in the stomach. The assessment of strain specific acid resistance properties and *in vitro* invasion capacity allows for an accurate and fast prediction of the infectivity of the different *Salmonella* strains. With the use of the parallelogram approach and acid survival capacity a realistic prediction can be made of the virulence of (new emerging) infective strains of *Salmonella* in human. This approach may also be valuable for prediction of virulence of other food-born pathogens.

ACKNOWLEDGEMENTS

We thank Prof. Dr. T. Abee for critically reading the manuscript and the Nutrition, Health, Safety and Sustainability program of the Netherlands Organisation for Health Research and Development for funding this research.

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CHAPTER 6

General discussion

P.A. Berk

Salmonella remains a very relevant health problem all over the world, in both developing and developed countries. *Salmonella* is not restricted by country boundaries and a number of 'outbreaks' that have occurred have involved more than one country. *Salmonella enterica* serovar Typhimurium phage type 104 (DT104) is a strain which emerged in the late 1980's[1]. This strain was found to be multi-antibiotic resistant and is resistant to at least 5 (but up to 9) different antibiotics. Again in 2005 an outbreak was observed through Europe, showing that *Salmonella* Typhimurium DT104 is still a very relevant problem for human health.

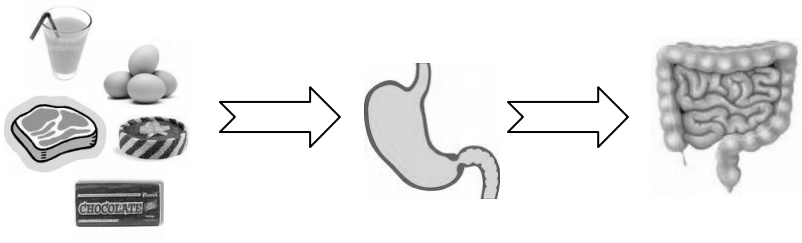
Salmonellosis outbreak linked to carpaccio made from imported raw beef

(Eurosurveillance Weekly 2005; 050922 and Eurosurveillance Weekly 2005;: 051201)
An outbreak of multiresistant ***Salmonella* Typhimurium DT104** infections associated with **beef** imported from Italy has recently occurred in **Denmark**. So far, **22** laboratory confirmed **cases** have been detected by the national passive laboratory surveillance system.

At the end of September 2005, an outbreak of ***Salmonella* Typhimurium DT104** infections was detected in **the Netherlands** by the Dutch National *Salmonella* Centre. From September to November **165 extra cases** of *S.* Typhimurium DT104 were recorded. The molecular typing indicated clonality of the outbreak isolates and suggested a link to a recent outbreak of *S.* Typhimurium DT104 in Denmark.

In this thesis the effect of growth phase and growth pH on the virulence of *S.* Typhimurium DT104 was investigated. Growth phase and growth pH can have effect:

in food, in the stomach and in the intestine.



A new tool, the parallelogram approach, was used to determine the probability of infection in humans of different *S.* Typhimurium DT104 strains. In addition it will be discussed whehter *S.* Typhimurium DT104 strains are more virulent than other *S.* Typhimurium or *Salmonella* strains.

GROWTH PHASE AND VIRULENCE OF SALMONELLA

The growth curve of bacteria is divided into 3 phases (Fig. 6.1). The lag phase encompasses the first part of the curve. During this time bacteria adapt to their new environment. The curve remains horizontal, since growth has not yet started or potentially balanced by early reproduction in some cells and death in others. The population then enters an active stage of growth called the exponential phase. The mass of each cell increases and reproduction follows. After this stage of growth, the vigour of the population changes and the population enters another plateau, the stationary phase. Bacteria stop reproducing because one of the nutrients is run out or by other inhibitory factors.

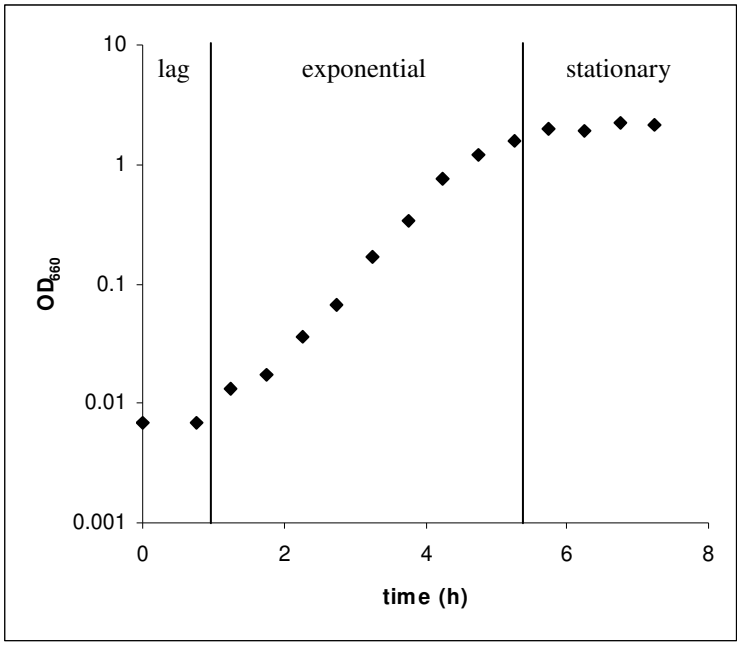
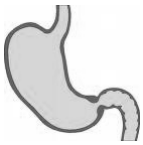
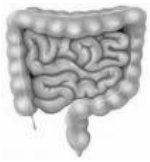


Figure 6.1 Growth phase of* Salmonella *Typhimurium DT104 strain 327 grown in LB medium with glucose in a closed flask without shaking at 37˚C.

It is not clear in what growth phase *Salmonella* is present in food products. It is likely that different scenario's can occur. Food products can be contaminated with *Salmonella* after preparation due to cross contamination with *Salmonella* contaminated raw products. If all nutrients are present in this food product and the food product is kept above the minimum growth temperature, *Salmonella* can grow and therefore will be in the exponential phase. *Salmonella* can also be in the exponential phase in food products if raw materials (e.g. eggs) used to make the food are contaminated with *Salmonella.* When the food product is stored improperly or re-contaminated (e.g. bavarois) and all nutrients are present, the *Salmonella* can grow. However when all nutrients for *Salmonella* are run out or there is an accumulation of inhibitory factors in the food product, *Salmonella* will enter the stationary phase. Since the environmental conditions are not optimal in most situations, there is also another possibility; *Salmonella* is fixated in a certain growth state. For example when meat is contaminated with *Salmonella*, the *Salmonella* can grow and will probably be in the exponential growth phase. However when the meat stored refrigerated or frozen the *Salmonella* will stop growing, but has not entered the stationary phase yet. When using a virulence assay it could be useful to use *Salmonella* in all three different stages to investigate effects of the environment since *Salmonella* from all three stages could respond differently. Also when setting food safety objectives it is important to determine in what growth phase bacteria are. However this is difficult to determine, the other possibility is to take a worst case scenario into account, for *Salmonella* one has to take in mind that *Salmonella* are more virulent in the exponential than in the stationary phase.



Due to the low pH *Salmonella* can not grow in the stomach. The growth phase of *Salmonella* can however be of importance in surviving the low pH of the stomach. *Salmonella* possesses different systems to survive low pH, some present in the exponential phase and some in the stationary phase. The exponential phase acid tolerance response of *Salmonella* is displayed in the exponential phase. With the use of this response *Salmonella* can survive at pH 3-3.5 for a prolonged period of time. In the stationary phase *Salmonella* possesses two systems which can protect them against low pH, the stationary phase acid tolerance response (ATR) and acid resistance (AR) systems. With the use of these systems in the stationary phase, *Salmonella* can survive a pH as low as 2.5. Since the pH of the stomach is very low, bacteria in the stationary phase have a higher change of survival than those in the exponential phase. In food products AR is likely to be of more importance than ATR, since the latter is determined in minimal medium[2] and food products are mainly rich of nutrients for *Salmonella.*



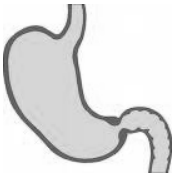
In this study it was shown that *Salmonella* are more virulent in the exponential phase than in the stationary phase in the intestines. *S.* Typhimurium DT104 strains showed higher invasive capacity in both human and rat cell lines (Chapter 3) and also a lower ID_{50} in rat (Chapter 4).This is in consistence with other studies, previous *in vitro* studies also revealed increased virulence of *Salmonella* exponential phase cells i.e. their invasion capacity was higher than that of stationary phase cells [3;4]. Increased virulence during exponential growth may be explained by the observation that genes involved in the adhesion process are maximally expressed during this growth phase. Hermans *et al.*[5] found in microarray studies using *S.* Typhimurium DT104 grown to different growth phases, that genes of the *Salmonella* Pathogenicity Island 1 (SPI-1) were up regulated at the end of the exponential phase. Several SPI-1 effector proteins are known to be involved in the invasion process of *Salmonella* (reviewed in [6]). In addition, Hermans *et al.* [5] have shown that LPS genes, which are thought to be important in the adhesion process, are also up regulated during exponential growth. Torres *et al.*[7] showed that the *lpf* (long polar fimbria) genes are maximally expressed during the mid exponential phase in Enterohemorrhagic *Escherichia coli*. In *Salmonella* the long polar fimbriae are found to mediate adhesion to M cells and the cells of the Peyers patches of the small intestine in a mouse model of infection [8]. They are therefore important at an early step of disease by an oral route of infection. It is conceivable that expression of *lpf* genes in *Salmonella* displays similar growth phase-dependent expression as in *E. coli*. However in this study no effect of growh phase was found on the adhesion of *S.* Typhimurium DT104 in the human intestinal cell line (Chapter 3).

ACID RESISTANCE AND VIRULENCE OF SALMONELLA

Acid resistance is an important mechanism of food borne pathogens to survive the acidic environment of the stomach and macrophages. *Salmonella* Typhimurium DT104 possesses different levels of acid resistance (Chapter 1), which can be induced by growth at pH 5. In our study we found large differences in acid resistance between the different *S.* Typhimurium DT104 strains, varying from less than 0.0001 % survival to 36 % survival after 2 hours at pH 2.5 (Chapter 2). Isolates from humans were more often highly acid resistant than isolates from foods, which indicates that strains that are acid resistance have a higher probability of causing an infection. This is logical since acid resistant strains can better survive the acidic environment of the stomach. Using the parallelogram approach in Chapter 5 it was shown that an adapted acid resistant strain has about 100 000 times higher probability of surviving the low pH of the stomach and thus of causing an infection in human than acid sensitive strains.

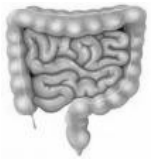
To become acid resistant bacteria have to be exposed to non-lethal low pH values. *S.* Typhimurium infections are mainly associated with the consumption of beef and pig meat. Several studies have shown that the feed for these animals contribute to the development of acid resistant bacteria. Pigs fed course nonpelleted feed showed increased numbers of anaerobic bacteria, increased concentrations of organic acids, and reduced pH in the stomach[9]. Also an increased *in vitro* death rate of *S. enterica* serovar Typhimurium DT12 in content from the stomach was shown. It was concluded that feeding a coarsely ground meal feed to pigs changes the physiochemical and microbial properties of content in the stomach which decreases the survival of *Salmonella* during passage through the stomach. Also for other bacteria an effect of feed on the stomach survival was found. Grain is often used as supplement feed for cattle since they grow faster and more efficiently than those fed grass or hay. However Russel *et al.*[10] have shown that grain feeding seems to promote the growth and acid resistance of *Escherichia coli* in fattening beef cattle and acid-resistant *E. coli* are more likely to survive the human gastric stomach. *E. coli* from colonic digesta from cattle fed hay were very sensitive to pH 2.0 acid shock. *E. coli* from colonic digesta from cattle fed grain were much more acid resistant, and a large proportion was able to survive the acid shock.

Another place where bacteria can become acid resistant is in the food product itself. Modern preservation techniques are becoming milder in response to the consumers' demands for higher quality, more convenient foods, which are less heavily processed, less heavily preserved, and less reliant on additive preservatives. However *Salmonella* have the potential to adapt to a wide variety of these mild stresses which can lead also to cross-protection against other stresses. In addition, the use of mild stresses can lead to selection of more resistant strains that will survive better in the minimal processed food product. Due to the ability of *Salmonella* to respond to modern preservation techniques a much higher risk exists of *Salmonella* surviving the food process and passing the stomach. To investigate whether a certain food product can be a risk for human health, the history of the food product has to be taken in account.



Acid resistance is an important virulence factor, since it increases the change of surviving the low pH of the stomach. But that does not mean that acid sensitive bacteria are harmless. Some food ingredients can protect the bacteria against the low pH of the stomach. Food-borne pathogens which are present in fatty food products like chocolate or peanut butter can escape the contact with the gastric juice by retaining in

fat particles and therefore survive the stomach passage. In June 2001 there was a large outbreak of *S.* Typhimurium DT104 from Helva, a Turkish dessert or sweet made from sesame seeds, in Sweden, Norway, Australia, Germany and Turkey [11]. It is assumed that the high fat fraction in the food matrix protected the bacteria against the gastric acid in the human stomach as shown previously for enteric pathogens [12]. Notably, a number of *S.* Typhimurium DT104 outbreaks have previously been associated with foods containing a high fat fraction [12-16]. Also ingestion of a meal can immediately raise the pH to about 5 or 6 [17 18]. During this period, potentially disease-causing bacteria can be transported to the small intestine without being destroyed by low pH. Takumi *et al.* [19] showed, by modelling the gastric pH and transport, that this was indeed the case for *E. coli*. For healthy young adults 20-73% of the ingested bacteria could survive the stomach and arrive in the small intestine.



In this study it was shown that once *S.* Typhimurium DT104 has passed the stomach, acid sensitive strains are as virulent as acid resistant strains. In *in vitro* studies with human and rat intestinal cells, acid sensitive strains have the same adhesive and invasive capacity as acid resistant strains (Chapter 3). Also in rat studies *in vivo*, acid sensitive strains were as virulent as acid resistant strains (Chapter 4).

INFECTIVITY IN HUMANS; THE PARALLELOGRAM APPROACH

Although *in vitro* experiments with human cell lines and *in vivo* experiments with rat can give some information about the infectivity of *Salmonella*, the goal is to determine the infectivity in humans. Therefore data from *in vivo* studies with rat and *in vitro* studies with human cell lines are used in a parallelogram approach to predict the infectivity in humans (Chapter 5).

In this approach (see Fig. 6.2) data derived from different *in vitro* cell systems can be compared with each other (A) and data from experiments with animal cells can be compared with *in vivo* animal data (B). Extrapolation can now follow two routes: from *in vivo* animal data to man (C) and from *in vitro* human data to man (D).

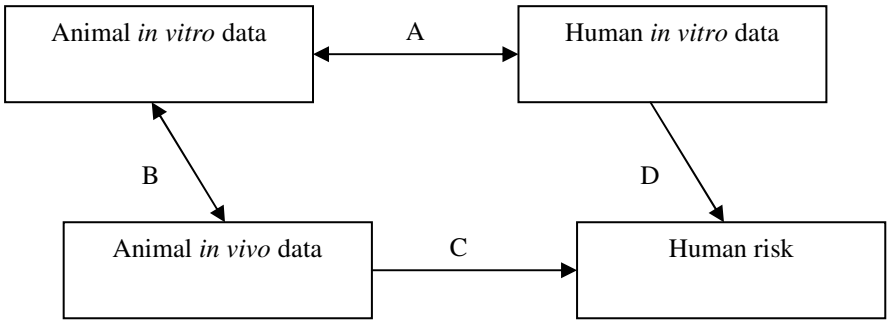


Figure 6.2. Parallelogram approach

In this thesis the log ID_{50} found for humans in this study following the parallelogram approach varies from 2.86 – 5.33. This is comparable to ID_{50} found in human feeding studies in the fifties (4.13-5.41).[20:21-23] In these studies healthy adult man were used an they are likely to are the least succeptible to infection with *Salmonella* and have probably high ID_{50} values compared to more succeptible individuals. From this study we can conclude that the invasive capacity *in vitro* of a certain *S.* Typhimurium DT104 strain culture under a certain condition is a good representation for the infectivity of this strain *in vivo*. Although this is the first time this kind of study is used for determining the probability of infection in humans, a few strains are tested and both growth pH and growth phase are studied. The large effects on virulence of growth phase and *S.* Enteritidis compared to *S.* Typhimurium DT104 strains grown to the stationary phase were found in both rat and human cell lines and also in the rat model. It would be useful to extend this study with more *Salmonella* strains from different serotypes with different invasive capacities to have a better description of the correlation between the intestinal cell lines and the rat experiments and to see if in general effects seen in human and animal cell lines are comparable to animal studies *in vivo.* To validate our model it would also be useful to include cell lines from different animals and animal studies. Stecher *et al.* [24] described a new mouse model with predominantly intestinal disease. These mice are either specific-pathogen-free mice pretreated with streptomycin to disrupt the intestinal microflora or germ-free mice, with no intestinal microflora at all.. Mice are relatively cheap and easy to use for these kinds of experiments and there are also intestinal cell lines from mice available. This could therefore be a useful addition to our parallelogram model. The most ideal situation is to use an animal which is closer related to humans, like pig for example. When this model is optimized in the future it can result in the reduction of animal models for testing of virulence of new (emerging) *Salmonella* or even other bacteria. By determining their invasiveness *in vitro* a good prediction can be made of their virulence in humans.

S. TYPHIMURIUM DT104 MORE VIRULENT?

One of the aims of this thesis was to investigate whether *S.* Typhimurium DT104 strains are more virulent than other *Salmonellas.* Therefore different virulence properties of *S.* Typhimurium DT104 strains were compared to that of an *S.* Enteritidis strain.
A large variation in level of acid resistance was found between the different *S.* Typhimurium DT104 strains. Although some strains were highly acid resistance this was certainly not the case for all *S.* Typhimurium DT104 strains.
In vitro the human intestinal cell line Caco-2 was used to test the virulence of different *S.* Typhimurium DT104 strains, which is described in Chapter 3. The adhesive capacity onto the intestinal cell line was comparable for the *S.* Typhimurium DT104 strains used and the *S.* Enteritidis strain. This was also the case for the amount of IL-8 produced by the intestinal cell line after infection with the *S.* Typhimurium DT104 strains and the *S.* Enteritidis strain. However, the invasive capacity of the *S.* Typhimurium DT104 strains was lower than that of the *S.* Enteritidis strain, which indicates that *S.* Typhimurium DT104 is not more virulent but less virulent than the *S.* Enteritidis strain used.
In vivo a rat model was used to study the infectivity of the different *Salmonella* Typhimurium DT104 strains (Chapter 4). The results of these *in vivo* experiments could be compared to that of Havelaar *et al*., who used the same rat model and the same *S.* Enteritidis strain that was also used in our *in vitro* experiments[25]. The dose at which 50 % of the rats are infected (ID50) is less for the *S.* Typhimurium DT104 strains used in this study than the *ID50* for the *S.* Enteritidis strain used in the study of Havelaar *et al*, [25]. However in the exponential phase the *S.* Typhimurium DT104 strains used appeared to be as virulent as the *S.* Enteritidis strain grown to the stationary phase. However this *S.* Enteritidis strain was not tested grown to the exponential phase in the study of Havelaar, thus no comparison could be made for bacteria grown to the exponential phase.

Summarizing: not all *S.* Typhimurium DT104 strains are highly acid resistant; the adhesive capacity and the induction of IL-8 production are comparable to that of an *S.* Enteritidis strain; the invasive capacity and the virulence in rat are lower than that of the same *S.* Enteritidis strain. However it can not be excluded that the *S.* Enteritidis strain used in this study is more virulent than other *S.* Enteritidis strains and therefor it is suggested to compare the virulence parameters of *S.* Typhimurium DT104 strains to different *S.* Enteritidis and *S.* Typhimurium strains. Taken this al together it can be concluded that the *S.* Typhimurium DT104 strains used in this study are not more virulent than a *S.* Enteritidis strain.

This is consistent with results from Allen *et al*. [26], who compared the ability of *S.*

Typhimurium DT104 strains to survive within murine peritoneal macrophages, the ability to invade intestinal cells (HEP-2), the resistance to reactive oxygen and nitrogen compounds and the ability to cause lethal infections in mice. Allen *et al*. also did not find any evidence for a higher virulence of *S.* Typhimurium DT104 strains. Also Threlfall *et al*.[27] did not find any evidence for increased virulence of *S.* Typhimurium DT104. The incidence of *S.* Typhimurium DT104 in blood isolates was comparable to that of other *S.* Typhimurium phage types and *S.* Enteritidis. Still the fact remains that some studies have shown that the clinical symptoms of multi-drug resistant *S.* Typhimurium DT104 are more severe than other *S.* Typhimurium or *S.* Enteritidis infections [28;29], which can not be explaned by resistant against antibiotics only.
Alternatively, up to now unknown factors may contribute to *S.* Typhimurium DT104 pathogenesis in the human host and the search for relevant *in vitro* and *in vivo* models needs to be pursued.

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SAMENVATTING

Salmonellose is de verzamelnaam voor een groep infectieziekten die worden veroorzaakt door bacteriën behorende tot het geslacht *Salmonella*. Besmetting geschiedt meestal door de consumptie van voedsel van dierlijke oorsprong (vlees, gevogelte, eieren, melk), maar ook andere voedselproducten, zoals chocola, zijn bekend als oorzaak van een *Salmonella* besmetting.
Salmonella is voor het eerst ontdekt en gerapporteerd in 1885 door D.E. Salmon . Op dit moment zijn vele soorten bekend, waar *S.* Typhimurium er één van is. *S.* Typhimurium kan onderverdeeld worden op basis van de gevoeligheid voor verschillende bacteriofagen. Eén van die faagtypes is *S.* Typhimurium DT104. In 1984 werd de eerste *S.* Typhimurium DT104 geïsoleerd die resistent bleek tegen 5 soorten antibiotica: ampiciline, chlooramfenicol, streptomicine, sulfonamide en tetracycline. Vanaf dat moment is dit multiresistente type veel gevonden in verschillende landen in en buiten Europa. Ook in Nederland is een snelle stijging van het aantal *S.* Typhimurium DT104 isolaten gevonden. In 2001 was 37.2 % van alle *S.* Typhimurium stammen van het faagtype DT104, waarvan op hun beurt weer 80.8 % resistent was tegen 5 of meer antibiotica.

Infecties door *S.* Typhimurium bij mensen kunnen verschillende ziektebeelden geven variërend van mensen zonder symptomen tot patiënten met maagdarm-stoornissen en symptomen zoals diarree (soms met bloed), hoofdpijn, koorts en misselijkheid. De symptomen beginnen ongeveer 12 tot 36 uur na consumptie en duren vaak ongeveer 5 dagen. Bij gezonde mensen gaat de ziekte vanzelf weer over en de enige behandeling die soms nodig is, is het voorkomen van uitdroging door vloeistof en elektrolyten toe te dienen. Sommige studies laten zien dat de symptomen van patiënten geïnfecteerd met de multiresistente *S.* Typhimurium DT104 ernstiger zijn dan de symptomen van andere *S.* Typhimurium infecties. Het is niet duidelijk of dit komt doordat *S.* Typhimurium DT104 andere ziekmakende eigenschappen heeft dan andere *Salmonella's* of dat *S.* Typhimurium DT104 de maag beter overleeft. Het doel van dit onderzoek is het bestuderen van het effect van omgevingsfactoren (met name zuur) op de ziekmakende eigenschappen van *S.* Typhimurium DT104. Daarnaast wordt geprobeerd een relatie te leggen tussen testen met darmcellen en testen met een heel organisme, om zo een voorspelling te kunnen doen van de situatie bij de mens.

De zure pH van de maag wordt gezien als de eerste barrière tegen voedsel-overdraagbare pathogenen. Zuurresistentie van pathogenen zou daarom bij kunnen dragen aan hun ziekmakende eigenschappen. In deze studie werden 37 *S.* Typhimurium DT104 stammen verzameld en getest op zuurresistentie **(hoofdstuk 2)**. Hierbij werd een grote variatie in

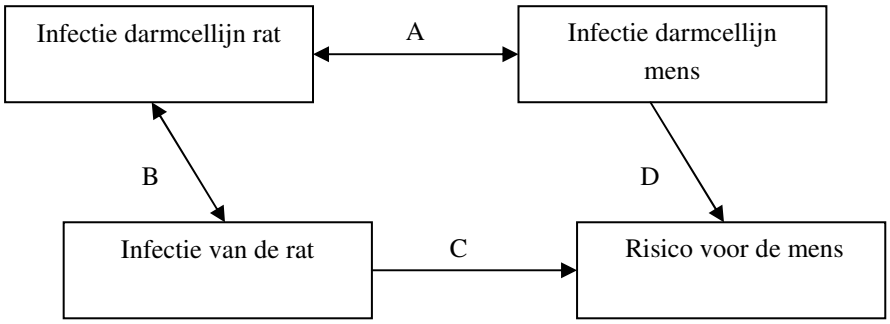
zuurresistentie gevonden tussen de verschillende *S.* Typhimurium DT104 stammen. Interessant is dat alle hoog zuurresistente stammen geïsoleerd waren uit patiënten, wat duidt op een positieve correlatie tussen zuurresistentie en kans op ziekte omdat ze een vergroot vermogen hebben om de maag te overleven. Echter ook andere factoren kunnen een rol spelen. *S.* Typhimurium DT104 stammen geïsoleerd uit Helva, een mild zuur voedselproduct, bleken laag zuurresistent. Toch waren deze stammen afkomstig uit een grote uitbraak in juni 2001 in Zweden, Noorwegen, Australië, Duitsland en Turkije. Waarschijnlijk heeft de hoge vetfractie in dit voedselproduct de bacteriën beschermd tegen het maagzuur en heeft zuurresistentie daarom mogelijk geen rol gespeeld. Twee zuurresistente (1 uit voedsel en 1 uit een patiënt) en twee zuurgevoelige stammen (1 uit voedsel en 1 uit een patiënt) zijn vervolgens geselecteerd om verder te bestuderen.

Allereerst is hiervoor een darmcellijn gebruikt om verschillende ziekmakende eigenschappen te bepalen **(hoofdstuk 3).** Deze eigenschappen waren: hechting aan de cellen, het binnendringen van de cellen en het stimuleren van het immuunsysteem. Om het aantal binnengedrongen bacteriën in de cellen (de invasie) te bepalen is het nodig om alle bacteriën aan de buitenkant van de cellen dood te maken. Normaal wordt hiervoor gentamicine gebruikt, maar omdat *S.* Typhimurium DT104 resistent is tegen veel antibiotica, waaronder gentamicine, kon dit niet gebruikt worden. Daarom moest er een ander antibioticum gevonden worden waar *S.* Typhimurium DT104 niet resistent tegen is en dat ook geen invloed had op de darmcellijn en de *Salmonella's* binnenin de darmcellen. Gentamicine in combinatie met ciprofloxacine voldeed aan deze eisen en is verder gebruikt in deze assay. Met het gebruik van deze assay kon worden aangetoond dat alle gebruikte *S.* Typhimurium DT104 stammen in dezelfde mate aan de cellen hechtten en binnendringen. Ook de inductie van de immuunrespons bleek hetzelfde voor de verschillende stammen. Ook het effect van groeicondities (het effect van groei pH) van de bacteriën op dezelfde parameters is getest. Voor alle stammen bleek dat groei bij een lage pH (pH 5) of een neutrale pH (pH 7) geen verschil uitmaakte voor de hechting, de invasie en de inductie van de immuunrespons. Snel groeiende bacteriën bleken beter de cellen binnen te dringen dan langzaam groeiende bacteriën terwijl er geen verschil werd gevonden in aanhechting en de inductie van de immuunrespons.

Ook in een rattenmodel is gekeken naar de ziekmakende eigenschappen van de verschillende *S.* Typhimurium DT104 stammen **(hoofdstuk 4).** In het eerste experiment is een standaardcurve gemaakt van één stam gekweekt bij een neutrale pH (pH 7). Deze curve is gemaakt door de de prevalentie van infectie in verschillende organen van de rat uit te zetten tegen de dosis. In het tweede experiment is de herhaalbaarheid van het gebruikte rattenmodel getest en deze bleek goed. Het aantal bacteriën in de verschillende delen van de

darm en in de milt bleken duidelijk afhankelijk van de dosis die de ratten toegediend hadden gekregen. Ook het aantal witte bloedcellen vertoonde een dosisafhankelijke stijging.
In het derde experiment zijn 1 zuurresistente en 1 zuurgevoelige stam gebruikt en het aantal bacteriën in de organen van de ratten is vergeleken met de referentiestam uit het eerste experiment. Er bleek geen verschil tussen de 3 gebruikte stammen. Ook groei bij een lage pH had geen effect (experiment 4) en was er geen verschil tussen een geneutraliseerde maag of een niet geneutraliseerde maag (experiment 5). In experiment 6 is gekeken naar het effect van groeifase. Van zowel van de zuurresistente als de zuurgevoelige stam bleken er meer bacteriën in de organen te vinden bij de ratten die geïnfecteerd werden met snel groeiende bacteriën dan bij ratten die geïnfecteerd werden met langzaam groeiende bacteriën. De dosis waarbij bij 50% van de ratten bacteriën in de organen terug gevonden werden was 30 keer lager bij snelgroeiende bacteriën dan bij langzaam groeiende bacteriën. Dit betekent dat er 30x minder bacteriën nodig zijn om 50 % van de ratten te infecteren. Dit effect was ook al te zien aan de stijging van het aantal witte bloedcellen van de rat op dag 2, terwijl dit bij de eerdere experimenten pas op dag 6 te zien was.

De resultaten van de darmcellijn en de resultaten van het rattenmodel zijn vervolgens gebruikt om met behulp van een parallellogram benadering de kans op infectie voor de mens te bepalen **(hoofdstuk 5)**.



De parallellogram benadering

Met deze benadering worden data van verschillende celsystemen vergeleken met elkaar (A) en data van een ratten darmcellijn vergeleken met data van de rat (B). Er kan nu op 2 manieren een vertaalslag worden gemaakt: van rat naar mens (aangenomen A = C) en van darmcellijn van de mens naar de mens (aangenomen B = D).
Beide routes van de parallellogrambenadering kunnen worden gebruikt. Bij ***route 1*** is er

een evenrechtige relatie bepaald tussen het binnendringen van *Salmonella* in de ratten darmcellijn en het binnendringen van *Salmonella* in de darmcellijn van de mens. Deze relatie is vervolgens gebruikt om de kans op infectie van 1 bacterie in de rat te vertalen naar de kans op infectie van 1 bacterie in de mens.
Bij ***route 2*** is er een relatie bepaald tussen het binnendringen van *Salmonella* in de rattencellijn en de kans op infectie van 1 bacterie in de rat. Deze relatie is vervolgens gebruikt om het binnendringen van *Salmonella* in de darmcellijn van de mens te vertalen naar de kans op infectie van 1 bacterie in de mens.
De kans op infectie in de mens is afhankelijk van de kans dat 1 *Salmonella* de mens infecteert en de gemiddelde dosis in de darm. Deze dosis is afhankelijk van de ingenomen dosis en het percentage *Salmonella's* dat de maag overleeft. In de rat kan aangenomen worden dat deze dosis gelijk aan de ingenomen dosis omdat de pH in de rattenmaag zo hoog is dat alle *Salmonella's* de maagpassage overleven. In de mens is de pH van de maag veel lager en met behulp van een zuurresistentietest van 2 uur bij pH 2.5 kan gekeken worden hoeveel *Salmonella's* de maag overleven. Van de zuurresistente *S.* Typhimurium DT104 overleeft ongeveer 36 % van de bacteriën de maag en van de zuurgevoelige *S.* Typhimurium DT104 overleeft slechts ongeveer 0.00027 % de maag (uitgaande van een pH in de maag van 2.5 en een verblijftijd van 120 min). De kans op infectie kan nu worden uitgerekend waarbij rekening wordt gehouden met de zuurresistentie van de *Salmonella's*. Uit deze berekening volgt dat zuurresistente stammen een bijna 100.000 maal hogere kans op het infecteren van een mens hebben dan zuurgevoelige stammen.

Concluderend kunnen *S.* Typhimurium DT104 stammen zeer zuurresistent zijn en lijken ze geen andere ziekmakende eigenschappen te hebben dan andere *Salmonellas.* De groeifase van de *Salmonella* lijkt van invloed op de ziekmakende eigenschappen. Snel groeiende bacteriën hebben een 30-60 maal hogere kans op het infecteren van een mens dan (bijna) uitgegroeide bacteriën. Als levensmiddelen in de koelkast worden bewaard kan het zo zijn dat bacteriën langzaam groeien terwijl ze nog lang niet uitgegroeid zijn. Omdat het onbekend de grotere kans op infectie komt doordat snelgroeiende bacteriën echt meer ziekmakend zijn of dat het komt doordat (bijna) uitgegroeide bacteriën minder ziekmakend zijn is het belangrijk om rekening te houden met de "worst case" bij het stellen van voedselveiligheidscriteria.
Het grootste effect op de ziekmakende eigenschappen van *Salmonella* lijkt de zuurresistentie. Zuurresistente stammen hebben een bijna 100.000 maal hogere kans op het infecteren van een mens dan zuurgevoelige stammen. Zuurresistentie kan verkregen worden door bacteriën doordat de zuurgraad in sommige voedselproducten vrij hoog is. Omdat de bacterie dan al gewend is aan zuur is het voor de bacterie makkelijker om de zure pH van de maag te overleven en een infectie te veroorzaken in de dunne darm.

DANKWOORD

Eindelijk is het zover, mijn proefschrift is helemaal afgerond. Dit proefschrift was echter niet tot stand gekomen zonder hulp van enkele mensen. Degenen die ik als eerst wil bedanken zijn mijn co-promotor Rob de Jonge en mijn promotoren Tjakko Abee en Marcel Zwietering. Rob, jij was mijn dagelijkse begeleider en ik wil je bedanken voor je enthousiasme in het begeleiden, maar ook je enthousiasme voor het onderzoek. Jouw deur stond altijd voor me open en ik heb veel gehad aan alle discussies die we hebben gevoerd. Tjakko, jij bent ook vanaf het begin bij het project betrokken geweest, je hebt het hele project in goede banen geleidt en ik heb veel gehad aan jouw kennis van microbiologie. Marcel, ik heb veel gehad aan jouw wiskundige kennis vooral bij de parallellogrambenadering kwam dit als geroepen. Daarnaast wil ik ook Jasper Kieboom, Armand Hermans en Henk Aarts bedanken voor hun aandeel in het ZonMW project. We hebben een goede samenwerking gehad ook al liepen onze onderwerpen toch af en toe redelijk ver uit elkaar en moest ik soms mijn best doen om alle moleculaire technieken en snufjes goed te begrijpen.

Tijdens mijn promotieonderzoek heb ik een aantal heel grote rattenproeven gedaan, de uitvoering van deze proeven was nooit gelukt zonder de heren en dame van de afdeling proefdierkunde bij het RIVM: Hans Strootman, Piet van Schaik, Dirk Elbers, Christine Soputan en Coen Molenbeek. Ik wil jullie bedanken voor alle inzet en de gezellige dagen tijdens de secties, ik heb veel van jullie geleerd tijdens deze dagen. Daarnaast wil ik ook Lisette de la Fonteyne en Yvonne Wallbrink bedanken voor alle hematologische analyses. Bij deze proeven zijn er naast de analyses in de sectie kamer en de hematologische analyses ook heel veel microbiologische analyses gedaan. En als ik zeg heel veel, dan bedoel ik ook heel veel. Ik wil dan ook John, Wilma, Ellen, Frans, Luc. Liesbeth en Michal bedanken voor hun hulp tijdens deze proeven, het was hard werken tijdens deze vaak lange, maar ook gezellige dagen. Daarnaast wil ik jullie ook bedanken voor de prettige en leuke samenwerking bij de levensmiddelen bacteriologie en hygiëne projectgroep. Frans jij was een fijne projectleider om mee te werken en John, Wilma, Ellen en Luc ik heb veel gehad aan jullie ervaringen en vond het fijn dat ik altijd bij jullie aan kon kloppen als ik praktische vragen had op het lab. Ook alle andere collega's bij MGB/LZO wil ik bedanken voor de leuke tijd. Ik ging altijd met erg veel plezier naar het lab en heb genoten van alle leuke discussies tijdens de koffiepauzes, de gezellige borrels, sportdagen en andere leuke activiteiten. Het is niet voor niets dat ik nog steeds bij LZO rondloop.

Laatst las ik ergens de volgende stelling: “Het is goed om het doen van (promotie)onderzoek af te wisselen met sporten, omdat sporten leidt tot lichamelijke inspanning en geestelijke ontspanning en het doen van onderzoek tot lichamelijke ontspanning en geestelijk inspanning” Ik ben het hier helemaal mee eens.
Ik wil dan ook iedereen bij acrobatiek bedanken voor de leuke, gezellige avonden, waarbij ik zowel inspanning, ontspanning en balans kan vinden. Daarnaast wil ik natuurlijk Majella dames 2 bedanken, mijn volleybalteam. Meiden, jullie zijn niet alleen mijn teamgenoten, maar ook goede vriendinnen geworden.

Naast werk en sport is mijn familie natuurlijk een belangrijk deel van mijn leven en zonder hun steun had ik dit proefschrift niet kunnen afronden. Lieve schoonfamilie, pap en mam, Marjolein en Martijn, Jeroen en Karin en Dennis, ondanks dat het soms lastig te begrijpen was wat ik eigenlijk allemaal aan het doen was deze periode, hebben jullie toch altijd veel interesse getoond in mij en mijn onderzoek en ik wil jullie dan ook bedanken voor al jullie steun. Pap en mam, jullie wil ik nog speciaal bedanken, jullie hebben mij altijd gestimuleerd het beste uit mezelf te halen en zonder jullie was ik dan nooit gekomen waar ik nu ben.

En als laatste wil ik natuurlijk Wijnand bedanken voor zijn onvoorwaardelijke en liefdevolle steun tijdens mijn promotieonderzoek. Ik weet dat ik niet altijd even makkelijk ben geweest tijdens deze periode. Maar jij bent altijd er altijd voor me geweest om me te steunen maar ook om te discussiëren over mijn onderzoek. We zijn samen naar Utrecht verhuisd om dichter bij mijn werk te wonen en we hebben hier samen een leven opgebouwd, wat ik niet meer zou kunnen missen.

CURRICULUM VITAE

Petra Angeli Berk werd geboren op 10 oktober 1977 in Amsterdam. Ongeveer 2 jaar later besloten haar ouders te verhuizen naar het stadje Vollenhove in Overijssel. In 1996 behaalde zij haar VWO-diploma aan het Zuyderzee College in Emmeloord. In dat zelfde jaar startte zij haar studie Biomedische Wetenschappen aan de Universiteit Leiden. Tijdens deze studie heeft zij drie korte stages gelopen. Bij de vakgroep Moleculaire Celbiologie over het onderwerp getiteld "Inactivering van het *vesicular stomatitis virus* (VSV) door de fotosensitizers Syslens B-Cl en Sylsens R en licht". Verder bij de afdeling Longziekten van het LUMC een klinische stage over het onderwerp getiteld "Biochemische effectiviteit van Apocynine in patiënten met emphyseem en bij de afdeling Maag-, Darm- en Leverziekten van het LUMC over het onderwerp getiteld "Clinical course of patients with Crohn's disease localised in the colon. Voor haar afstudeerstage is zij buiten de Universiteit Leiden gaan zoeken en kwam terecht bij het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) bij het Microbiologische Laboratorium voor Gezondheidsbescherming (MGB), waar zij haar afstudeerstage heeft afgerond getiteld "The influence of growth pH and gastric pH shock on the immune response and invasiveness of *Salmonella enterica* serovar Enteritidis in human epithelial Caco-2 cells". Daarnaast heeft zij haar afstudeerscriptie getiteld "De pathogenese van *Campylobacter*" geschreven. In april 2001 rondde zij haar universitaire studie af, waarna zij in september van datzelfde jaar begon met haar promotieonderzoek bij hetzelfde laboratorium van het RIVM in samenwerking met de leerstoelgroep Levensmiddelenmicrobiologie van de Wageningen Universiteit. Het onderwerp van haar promotieonderzoek was het bestuderen van stress-response en virulentie van *Salmonella* Typhimurium DT104 in cellijnen en een diermodel. Dit promotieonderzoek werd begeleid door Dr. Rob de Jonge, Prof. Dr. Ir. Marcel Zwietering en Prof. Dr. Tjakko Abee en heeft geresulteerd in dit proefschrift. Vanaf oktober 2005 is Petra werkzaam als onderzoeker bij het Microbiologisch Laboratorium voor Gezondheidsbescherming (MGB) van het Centrum voor Infectieziektenbestrijding (Cib) bij het RIVM, wat sinds januari 2007 haar naam heeft veranderd in Laboratorium voor Zoonosen en Omgevingsmicrobiologie (LZO).

Cover Design: Jeroen Berk
Printed by: Ponsen & Looijen B.V.