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Dose response relationships for gastro-intestinal pathogens in an animal model

AH Havelaar, J Garssen, K Takumi, M. Koedam,
JB Dufrenne, FM van Leusden, L de la Fonteyne,
JG Vos

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RIVM, P.O. Box 1, 3720 BA Bilthoven, telephone: 31 - 30 - 274 91 11; telefax: 31 - 30 - 274 29 71

Abstract

Although dose–response models play an essential role in quantitative microbiological risk assessment, appropriate available data for this assessment are scarce. Since human volunteer studies provide only limited information, an animal model is being developed to study infection with human enteropathogenic bacteria. The model used adult, male WU rats exposed to different doses of three enteropathogenic bacteria (*Salmonella enterica* serovar Enteritidis, *Escherichia coli* O157 and *Campylobacter jejuni*) after overnight starvation and neutralisation of gastric acid. A relationship between the dose and the probability of infection was demonstrated for all three bacteria, with *C. jejuni* being more infectious than *E. coli* O157 and *S.* Enteritidis the least infectious of all three. *S.* Enteritidis induced a systemic infection, and dose-dependent effects were observed for some haematological parameters (notably an increase in neutrophils), as well as for histopathological changes in the gastro-intestinal tract and the delayed-type hypersensitivity reaction. The experiments using *S.* Enteritidis showed good reproducibility, whereas the reproducibility for the experiments with *C. jejuni* was poor.

Preface

The experiments in this report involved the work of many persons in different laboratories. Hans Strootman, Mariska van Dijk, Dirk Elberts and Bert van Middelaar (CDL) were responsible for animal experiments. Coen Moolenbeek performed section of the animals and Paul Roholl (LPI) was responsible for histotechnique, Henny Loendersloot and Sandra de Waal performed the histotechnical work. Yvonne Wallbrink (LPI) participated in haematological analyses. Ellen Delfgou-van Asch and Wilma Ritmeester (MGB) were involved in the microbiological analyses. Wim Jansen and Nan van Leeuwen provided bacterial strains. Joke van der Giessen (MGB), Marion Koopmans (LIO), Joop Schellekens and Ron Boot (LIS) provided helpful advice on biomedical aspects, and Wout Slob and Peter Teunis on statistical aspects of the work.

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Samenvatting

Dosis-respons modellen zijn een belangrijk onderdeel van de kwantitatieve microbiologische risicoanalyse, maar er zijn slechts weinig bruikbare kwantitatieve gegevens. Experimenten met vrijwilligers geven slechts beperkte informatie. Daarom wordt een diermodel voor infectie met enteropathogene bacteriën ontwikkeld. In een voorgaand rapport (Garssen *et al*., 1998) werd beschreven dat drie enteropathogene bacteriën (*Salmonella enterica* serovar Enteritidis, *Escherichia coli* O157, en *Campylobacter jejuni*) in staat waren het darmkanaal van volwassen, mannelijke WU ratten te koloniseren na besmetting van de dieren met hoge aantallen bacteriën (10^9-10^{10} cfu/dier) via een maagsonde. De kolonisatie werd bevorderd door overnacht vasten en neutralisatie van maagzuur met natriumbicarbonaat.
In dit rapport wordt onderzoek beschreven naar dosis-responsrelaties in dit rattenmodel. Daartoe werden de dieren besmet met verschillende aantallen bacteriën (10^1-10^{10} cfu/dier), onder overigens gelijkblijvende omstandigheden. De drie geteste enteropathogene species waren alle in staat het maag-darmkanaal van de rat te koloniseren, in tegenstelling tot de negatieve controlestam *E. coli* WG5. Na toedienen van hoge doses veroorzaakte *S*. Enteritidis ernstige systemische ziekte, maar *C. jejuni* en *E. coli* O157 koloniseerden het darmkanaal zonder klinische effecten te veroorzaken. Een relatie tussen de kans op infectie en de dosis werd gevonden voor iedere bacteriesoort, waarbij *C. jejuni* het meest infectieus was, gevolgd door *E. coli* O157 en *S*. Enteritidis. Met *S*. Enteritidis werd ook een dosis-responsrelatie gevonden met sommige hematologische parameters (met name een toename van neutrofielen), met de vertraagd type overgevoeligheidsreactie (OTV) en met histopathologische veranderingen in het darmkanaal. Bij blootstelling van de dieren aan lage aantallen *S*. Enteritidis werd geen fecale uitscheiding gevonden maar wel systemische infectie, zoals bleek uit isolatie van de bacteriën uit de mesenteriale lymfklieren en uit de milt. De positieve OTV reactie duidt op inductie van (systemische) cellulaire immuniteit tegen de bacterie.

Summary

Dose-response models are an essential part of quantitative microbiological risk assessment, but not many appropriate quantitative data are available for this purpose. Human volunteer studies provide only limited information. Therefore, an animal model for infection with human enteropathogenic bacteria is being developed. A previous report (Garssen *et al*., 1998) describes that three enteropathogenic bacteria (*Salmonella enterica* serovar Enteritidis, *Escherichia coli* O157, and *Campylobacter jejuni*) were able to colonise the intestinal tract of adult, male WU rats after inoculation by gavage of high doses (10^9-10^{10} cfu/animal). Overnight starvation and neutralisation of gastric acid with sodium bicarbonate promoted colonisation.
This report describes investigations in dose-response relations using the rat model. Animals were inoculated with different doses of bacteria (10^1-10^{10} cfu/animal), under similar conditions. The three enteropathogenic species were able to colonise the rats' intestinal tract, in contrast to the negative control strain *E. coli* WG5. When high doses were administered, *S.* Enteritidis induced severe systemic effects, but *C. jejuni* or *E. coli* O157 colonised the intestinal tract without signs of clinical illness. A relationship between the dose and the probability of infection was demonstrated for all three bacteria, with *C. jejuni* being more infectious than *E. coli* O157 and *S.* Enteritidis being least infectious. For *S.* Enteritidis, a dose-response relation was also demonstrated for some haematological parameters (notably an increase in neutrophils), for delayed type hypersensitivity (DTH) reaction and for histopathological changes in the intestinal tract. Animals challenged with low doses (300 cfu/animal) of *S.* Enteritidis did not shed the bacteria in their faeces, but systemic infection was demonstrated by isolation of the bacteria from mesenteric lymph nodes and spleen. The positive DTH reaction indicated induction of (systemic) cellular immunity against *S.* Enteritidis.

1. General introduction

Microbiological risk assessment is an emerging tool for the evaluation of the microbiological safety of food and water, and is increasingly used by regulatory agencies, both national and supranational. In this process, hazardous micro-organisms are identified and exposure of the consumer to these organisms is estimated by a combination of observational data and mathematical modelling. The health risk arising from exposure is then estimated by use of a dose-response model, that gives a quantitative description of the relationship between the exposure to a certain number of pathogens (the dose) and the probability of an effect, such as infection or disease.

Dose-response models can be based on observational or experimental data. Observational data (usually from food- or waterborne outbreaks) have the advantage that they are based on actual situations, but are extremely limited in availability for several reasons. The dose may be too low to be measured accurately, such as in drinking water, the contamination may be rare, such as with foodstuffs, or samples of the causal product may simply not be available. Furthermore, the size of the exposed population is often not known. Experimental data have the advantage that they are obtained under well-controlled conditions and can therefore be subjected to detailed mathematical analysis (Teunis *et al*., 1996). Typically, healthy volunteers are exposed to different doses of enteric pathogens that have been maintained and cultured in laboratory conditions. The volunteers are then closely monitored for signs of infection (excretion, seroconversion) and symptoms of illness. The limitations of the experimental approach follow from the experimental set-up: for ethical reasons, the exposed population is limited to healthy, usually adult, volunteers and the range of micro-organisms that can be tested is limited to those which are known to induce mild, self-limiting disease only. Furthermore, the laboratory-adapted cultures may not be representative of the micro-organisms as they occur in nature.

To overcome some of the limitations of the experimental approach in man, we aim at the use of animal models for infection, and possibly disease, by enteric pathogens. Such models should enable us to evaluate the effect of single, host- or pathogen-related factors on the pathogenesis, and to make inferences about dose-response relations in humans. Typical questions are: what is the effect of factors such as age, immunological status, non-specific barriers (e.g. gastric acid) on the susceptibility of the host, and factors such as bacterial stress-proteins, protection by fatty substrates etc. on the infectivity of the pathogen. As a next step, we plan to develop kinetic models of the infection process, that describe the dynamics of the host-pathogen interaction in the alimentary tract, for which the animal models should provide insight in important mechanisms and parameter estimates.

In a first series of experiments (Garssen *et al*., 1998), it was concluded that, after neutralisation of gastric acid with sodium bicarbonate, young adult male WU rats were susceptible to oral infection with high doses (10^9-10^{10} cfu per animal) of three enteropathogenic bacteria: *Escherichia coli* O157, *Salmonella enterica* serovar Enteritidis and *Campylobacter jejuni*. Subsequent pathological changes differed markedly for each pathogen. *S*. Enteritidis was highly invasive, resulting in systemic infection and sometimes death. *C. jejuni* led to pathological changes indicative of enteritis in some animals, and to systemic effects on blood parameters. *E. coli* O157 infection was restricted to the intestines and did not produce marked pathological changes.

In this report, we describe the effect of different doses of the three enteropathogenic bacteria on infection and disease in rats, and the reproducibility of the observed effects.

2. Susceptibility of rats to different doses of enteropathogenic bacteria by oral exposure

2.1 Introduction

To evaluate dose-dependent effects of exposure of adult rats to three enteropathogenic bacteria (*Salmonella enterica* serovar Enteritidis, *Escherichia coli* O157 and *Campylobacter jejuni*), the animals were inoculated with different doses between 10^1 and 10^{10} cfu per animal under the same experimental conditions as previously reported (Garssen *et al*., 1998).

2.2 Materials and methods[1]

2.2.1 Animals

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

2.2.2 Bacterial strains

Escherichia coli, strain number 30 serotype O157 K- H-, an isolate from a patient with Haemolytic Uremic Syndrome with *eae* and *stx2* genes;
Salmonella enterica serovar Enteritidis, strain number 97-198, patient isolate (origin RIVM);
Campylobacter jejuni, strain number B258, serotype O59 (from chicken faeces, Medema *et al*., 1992); and
E. coli, strain number WG5 (a nalidixic acid resistant derivative of *E. coli* C; Havelaar and Hogeboom, 1983) was used as a negative control.
From all strains a -70 °C collection was made by pure culturing on Brain Heart Infusion (BHI) agar (18-20 h at 37 °C) and inoculating a single colony in BHI, incubated for 18-20 h at 37° C (*C. jejuni* was incubated under micro-aerophilic conditions at 37° C for 48 h). After incubation, 0.1 ml of the culture was added to cryotubes filled with glass beads and 0.1 ml of glycerol. Directly after adding the cultures the cryotubes were placed in a -70 °C freezer.

2.2.3 Inoculum cultures

Both *E. coli* strains and *S.* Enteritidis were inoculated from the -70° C collection in BHI and incubated at 37° C for 18 h. After incubation, 100 ml of each culture was centrifuged at 5000 x *g* for 10 min at 4 °C. The supernatant was discarded and the pellet was re-suspended in 100 ml physiological saline (PS), followed by re-centrifugation. Again, the supernatant was discarded and the pellet was re-suspended in a volume of 4 ml PS. The cell suspension and serial (1:100) dilutions in PS were delivered at the animal department on melting ice. *C. jejuni* was treated similarly, but incubated under micro-aerophilic conditions at 37° C while shaking at 100 rpm for 48 h. For washing and concentrating, the culture was filtered through an 0.22 µm membrane filter (Nalgene 115 ml) instead of centrifugation.

[1] This experiment is registered as AAP/199800164

Directly before administration to the animals, 4 ml of each bacterial suspension was mixed with 4 ml of a solution of 6% (w/v) $NaHCO_3$. After administration (2.2.4), the inoculum cultures were transported to the microbiological laboratory on melting ice for plate counts on sheep-blood agar (incubated as above).

2.2.4 Experimental design

Each animal was implanted with a temperature transponder (BioMedic Data Systems, Seaford, Delaware, UK), subcutaneously in the neck. The transponder was coded for the (unique) animal number and was used for body temperature detection each day around 9.00 am. Body temperature and animal numbers were registered using a Biomed Pocket Scanner from Plexx, Elst, The Netherlands.
After one week of rest (i.e. acclimatisation), the animals were starved overnight (water *ad libitum*). After 16 hours of starvation, 1 ml of the bacterial suspensions was orally administered by gavage (3 animals per group, 5 groups per pathogen). Directly after gavage (day 0) food and water was provided *ad libitum*.
Blood samples were taken via orbita plexus puncture using a capillary under light ether anaesthesia on days –1, 3 and 6 (*S.* Enteritidis and *E. coli* O157 experiments). In the *C. jejuni* study, blood samples were obtained on days –1, 5 and 11. Approximately 1 ml of blood was obtained per sampling time point.
Daily clinical observations were made with reference to the status of general health of the animals. Special attention was paid to the nature of the faeces. The animals were weighed each day (early in the morning) starting one day prior to the oral inoculation. Each morning faeces was obtained directly from each rat in each group. The faeces was macroscopically evaluated and tested for microbiology the same day (in weekends – days 1 and 2 – samples were stored at 4 °C and examined the next Monday – day 3).
The animals were sacrificed on day 6 (day 11 for *C. jejuni*) after oral inoculation, by bleeding from the abdominal large vessels under nembutal anaesthesia (i.p.). The caecum of each rat was weighed after euthanasia and caecum weights relative to bodyweight were calculated. Urine was obtained from the bladder and stored at -80 °C. The entire experiment was divided into three cohorts, each cohort with its own negative control (WG5).

2.2.5 Faecal moisture content

As an indicator for diarrhoeal-like effects, the moisture content of the faeces was determined each day by calculation from the weight loss after drying. For this purpose, the samples were weighed in a glass container with airtight lid and dried in the open container for 1 hour at 103-105 °C. After cooling in an exsiccator with silica-gel, the container was closed and weighed. This procedure was repeated several times until constant weight.

2.2.6 Haematology

As an indicator for (systemic) infection, haematology for each rat was performed in blood samples, anti-coagulated with K_2EDTA, obtained at day -1 and 6 or 11. The haematological analyses were performed using the H1-E, a multi-species haematology analyser (Bayer B.V., Mijdrecht, The Netherlands) with multi-species software, version 3.0.

The following parameters were determined:
- Haemoglobin concentration (Hgb)
- Haematocrit (Hct)
- Red Blood cell Concentration (RBC)
- Mean Cell Volume (MCV)
- Mean Cell Haemoglobin (MCH)
- Mean Cell Haemoglobin Conc. (MCHC)
- Red blood cell Distribution Width (RDW)
- Haemoglobin Distribution Width (HDW)
- Platelets (Plt)
- Mean platelet volume (MPV)
- White Blood cell Concentration (WBC)
- Differentiation of white blood cells (% and absolute numbers)

2.2.7 Microbiology

Faecal samples were homogenised and diluted 1:10 (w/v) in peptone-physiological saline (PPS) and appropriate dilutions were spread on Brilliant Green Agar (BGA) for *S. enteritidis*, on Sorbitol McConkey cefixime-tellurite agar (SmacCT) for *E. coli* O157, on Tryptone Yeast Extract Glucose agar with nalidix acid 100 μg/ml (TYGnal) for *E. coli* WG5 and on Bolton medium for *C. jejuni* B258. The BGA, SmacCT and TYGnal were incubated at 37° C for 22-26h, 18-20 h and 18-20h, respectively. The Bolton medium was incubated under micro-aerophilic conditions for 44-48h at 42° C.
Mesenteric lymph nodes were homogenised in PPS by using an Ultra Thurrax. Appropriate dilutions were spread-plated on BGA, SMAC CT, TYGnal and Bolton medium and incubated as described above. Blood samples (day 3) were analysed by spread-plating 0.1 ml volumes on the same media.

2.2.8 Pathology

Immediately after exsanguination the abdomen and thorax were inspected and the gastro-intestinal tract was removed and processed. Macroscopic abnormalities were recorded as well as gross data per intestinal segment (volume and outer colour, gut associated lymphoid tissue, quantity and quality of content, thickness of wall, aspect of mucosa). The stomach, 3 segments of the small intestine (duodenum, jejunum and ileum, each with Peyers' patch), caecum, proximal and distal colon, mesenteric lymph nodes, liver, spleen and tissue of macroscopically abnormal organs were sampled and fixed in 3.8% phosphate buffered formaldehyde. The gut segments were processed using the Swiss roll technique (Moolenbeek and Ruitenberg, 1981). After fixation the tissues were embedded in paraplast and 4-5 μm thick sections were prepared and routinely stained with haematoxylin and eosin (HE, all dose groups) and PAS (control and highest dose group). Histopathological examination was confined to the gut. Individual data were tabulated manually. Only well-cut parts of sections were scored. If oedema was present in the outer winding of the Swiss roll only, this was considered an artefact. Histopathological examination was performed without knowledge of the treatment.

2.2.9 Statistical analysis

Analysis was done using Mathematica version 4.0 and Microsoft Excel. p-values less than 5% were considered significant. The development of body temperature and faecal moisture content were assumed to be proportional to days, and were analysed with linear regression models. We fitted the model for body temperatures to the data obtained from day –2 to day 6

(*S.* Enteritidis), or to day 11 (*C. jejuni*). The data for faecal moisture content were used from day –1 to day 6 (*S.* Enteritidis), to day 5 (*E. coli* O157), or to day 10 (*C. jejuni*). Welsh's approximate test was applied to compare the relative caecum weight of each of the groups with the control group. We analysed the haematological data for each of the bacterial infections by means of linear regression using the following model:

$$\mathrm{Log}_{10}(\mathrm{RESPONSE}) = \alpha + \beta\ \mathrm{DAY} + \gamma\ \mathrm{DAY}\ \mathrm{Log}_{10}(\mathrm{DOSE})$$

The variable DAY means either prior to infection (DAY = 0) or post infection (DAY = 1). For the Log_{10}(DOSE) term, we used a value of 0 for the control group. In this model, a significant day-effect due to experimental conditions is observed if $\beta \neq 0$ and a dose-related effect if $\gamma \neq 0$.

2.3 Results

2.3.1 Inoculum cultures

Table 1 shows the plate counts of different dilutions of the inoculum culture on a $\log_{10}$ scale. The intended 1:100 dilution ratio was obtained, with the exception of the very low doses for *S.* Enteritidis and *C. jejuni*.

Table 1. Microbiological analysis (colony count per ml, $\log_{10}$ in brackets) of inoculum cultures

Dose group	*S.* Enteritidis	*E. coli* O157	*C. jejuni*
Highest	2.8×10^9 (9.4)	4.6×10^9 (9.7)	2.7×10^9 (9.4)
Second highest	3.9×10^7 (7.6)	4.7×10^7 (7.7)	2.4×10^7 (7.4)
Intermediate	3.3×10^5 (5.5)	3.9×10^5 (5.6)	1.2×10^5 (5.1)
Second lowest	4.7×10^3 (3.7)	5.7×10^3 (3.8)	1.1×10^3 (3.0)
Lowest	1.5×10^1 (1.2)	5.0×10^1 (1.7)	7.0×10^1 (1.8)
Control (WG5)	3.2×10^9 (9.5)	1.4×10^9 (9.1)	5.3×10^9 (9.7)

2.3.2 Clinical observations

Two out of three *Salmonella* infected animals (highest dose) showed severe signs of illness (low muscle tension, cold, increased nasal discharge, red crusts around eyes and nose tips, ruffled fur) at days five and six after infection. In most groups, the body weight increased after inoculation (see Figure 1). In the *S.* Enteritidis highest dose group, the rats lost weight.

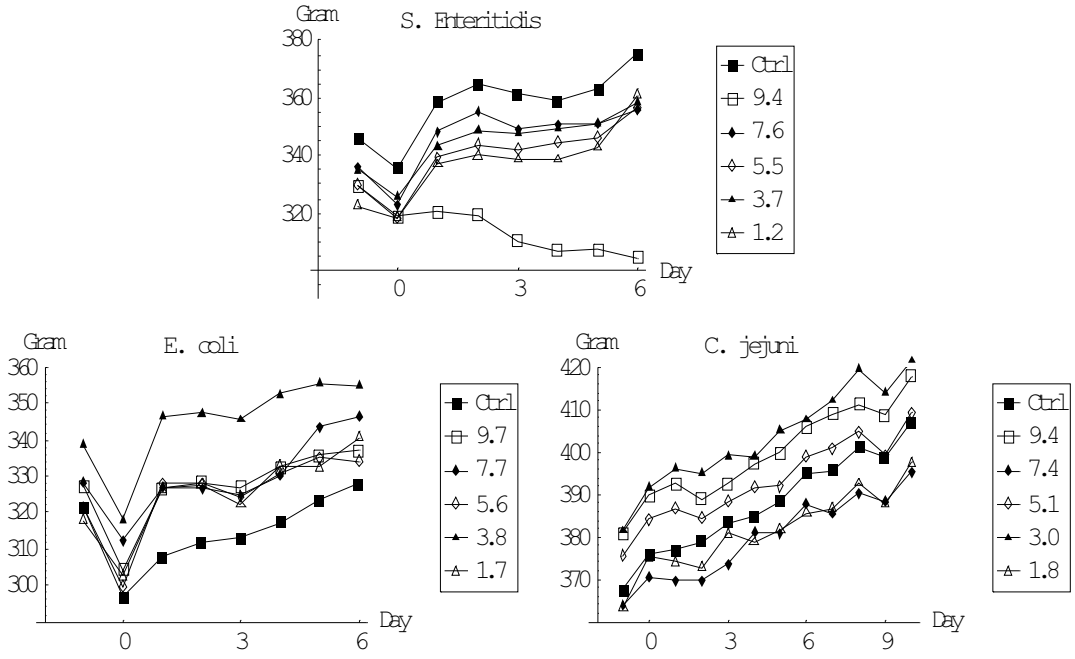

Figure 1. Body weights of adult, male WU rats after oral exposure to three enteropathogenic bacteria. Each symbol indicates the mean of 3 individual animals. Legend: $\log_{10}$ dose per animal, Ctrl: control (WG5).

Body temperatures and faecal moisture content did not show consistent trends over time in relation with infection.

The caecum weights in all animals were less than 2% of the total body weight, indicating no major abnormality in the rat's intestinal micro-flora. Compared to the control groups, there was a significant difference in caecum to body weight ratio in some groups (*S.* Enteritidis highest dose group, *E. coli* O157 second highest and second lowest dose groups, see Figure 2).

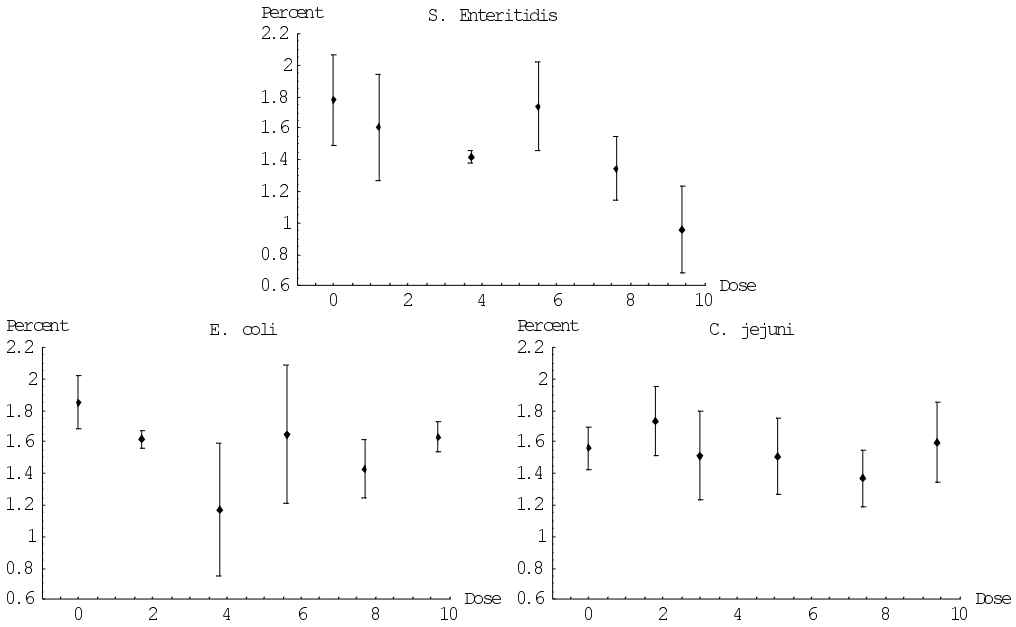

Figure 2. Caecum to body weight ratio of adult, male WU rats after oral exposure to three enteropathogenic bacteria. Each symbol indicates the mean and standard deviation of 3 individual animals. Dose: log_{10} dose per animal, 0: control (WG5).

2.3.3 Microbiology

No *E. coli* WG5 was detected in any of the faecal samples, blood or lymph nodes. Figure 3 shows the faecal excretion of pathogens in relation to dose and time after inoculation. For all pathogens, the excretion pattern was dose-related and in general higher numbers were excreted after exposure to higher doses. Exposure to *S.* Enteritidis resulted in a sharp increase of faecal counts, followed by a transient decrease and subsequent increase. This could indicate initial attachment of bacteria to the intestinal epithelia, followed by gradual detachment and active growth from day 3 onwards. Inoculation with *E. coli* O157 also induced a steep increase in faecal counts, followed by a relatively slow decrease. This could also indicate initial attachment and subsequent detachment at a lower rate than do *S.* Enteritidis. Only in the second lowest dose group was there some indication for growth: in one animal the faecal counts increased after day 3 but only to low levels. After exposure to *C. jejuni*, a less pronounced initial increase of faecal counts was observed. At higher doses, the faecal counts increased from day 1 onwards, whereas at lower doses a lag-period was observed. In all dose-groups, the counts increased to reach a level of 10^5-10^6 per gram after 7-11 days, independent of the initial dose. This pattern might be explained by initial die-off, followed by active growth of the surviving bacteria.

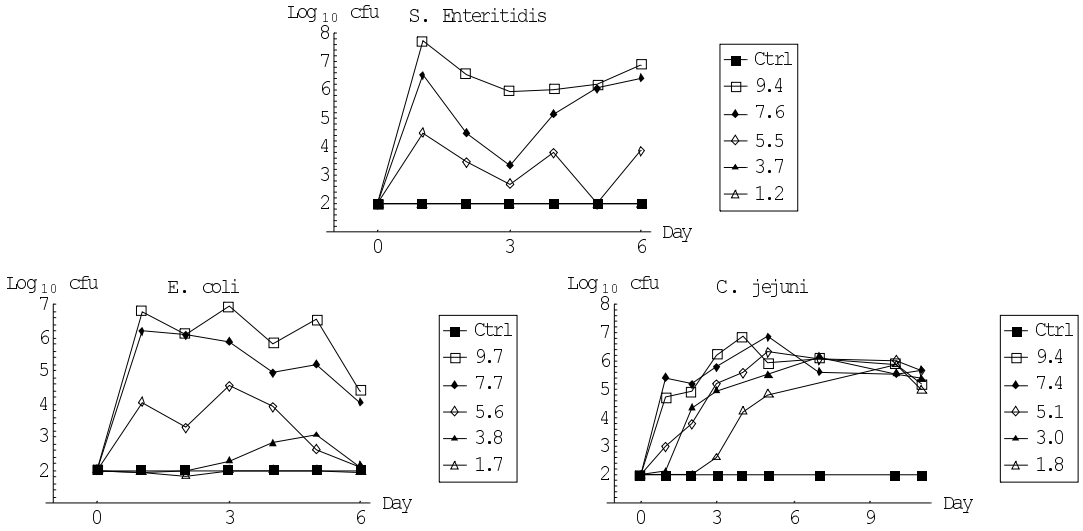

Figure 3. Colony counts in the faeces of adult, male WU rats after oral exposure to three enteropathogenic bacteria. Each symbol indicates the mean of 3 individual animals. Legend: log_{10} dose per animal, Ctrl: control (WG5).

For purposes of dose-response modelling and comparison with human volunteer experiments, it is also of interest to interpret the data based on two outcomes (the animal was infected or not infected). For this purpose, infection can be defined as equivalent to faecal shedding at any moment in time (note that in the control groups no bacteria were detected in faeces at any moment). In lower dose-groups, however, counts of 50 cfu per gram (i.e. one specific colony on one of two duplicate plates) were sometimes seen and these could be attributed to transient colonisation only. Therefore, we have adopted a somewhat higher threshold to define infection: faecal excretion of ≥ 1000 cfu per gram on at least two days as the criterion for infection (i.e. active multiplication of the pathogen in the gastro-intestinal tract). The results can then be summarised as in Table 2. It shows that *C. jejuni* was the most infectious of the three pathogens tested, followed by *E. coli* O157 with *S.* Enteritidis being least infectious.

Table 2. Infection (defined by faecal excretion) of adult, male WU rats after oral exposure to three enteropathogenic bacteria

Dose group	Rats infected/exposed		
	S. Enteritidis	*E. coli* O157:H7	*C. jejuni*
Highest	3/3	3/3	3/3
Second highest	3/3	3/3	3/3
Intermediate	3/3	3/3	3/3
Second lowest	0/3	2/3	3/3
Lowest	0/3	0/3	2/3

No pathogens were detected in any of the blood samples. The counts in mesenteric lymph nodes are shown in Table 3. For *S.* Enteritidis, these data confirm the observations in the faeces that infection occurred in the three highest dose-groups, but not in the two lowest groups. The mean count in mesenteric lymph nodes increased with increasing dose. *E. coli* O157 was not detected in mesenteric lymph nodes, and *C. jejuni* only in low numbers. Not all animals that were excreting *C. jejuni* had positive cultures in mesenteric lymph nodes.

Table 3. Pathogenic bacteria (cfu per gram) in mesenteric lymph nodes of adult, male WU rats after oral exposure to different doses

Dose group	*S.* Enteritidis (day 6)	*E. coli* O157 (day 6)	*C. jejuni* (day 11)
Highest	3.7×10^5 (3)	- (0)	- (0)
Second highest	1.4×10^5 (3)	- (0)	1.2×10^3 (1)
Intermediate	6.6×10^4 (3)	- (0)	8.0×10^2 (1)
Second lowest	- (0)	- (0)	- (0)
Lowest	- (0)	- (0)	1.1×10^3 (1)

Each data point indicates the mean of positive animals. Shown between brackets is the number of positive animals out of three. Dash (-): all animals below detection level (100 cfu/g)

2.3.4 Haematology

Detailed information on the results of haematological analysis is given in Figure 4-Figure 6. Table 4 shows a summary of significant changes in the blood parameters. With one or more pathogens, there was a significant decrease in the baseline level of several parameters related to red blood cells (Hgb, MCH, MCHC, and RDW; note an increase with *C. jejuni* for the latter parameter). This might indicate anaemia induced by experimental conditions, which could be related to the repeated sampling of blood from the animals (at day –1, at day 3 or 5 and at day 6 or 11 at autopsy). The baseline level of several white blood parameters (WBC, monocytes, lymphocytes, basophils, LUCs) also decreased with one or more pathogens. A dose-dependent increase in the number of cells involved in inflammatory reactions (neutrophils and monocytes) was observed in animals exposed to *S.* Enteritidis, indicating severe systemic effects of these bacteria. In the *S.* Enteritidis exposed animals, there was also a dose-related decrease of MCV and MCH. This might indicate that the overall reduction of haemoglobin by experimental conditions is enhanced by exposure to *S.* Enteritidis (possibly by less efficient supplementation). No systemic effects were observed after exposure to *E. coli* O157 or *C. jejuni*.

Table 4. Changes in haematological parameters in adult, male WU rats after oral exposure to three enteropathogenic bacteria

	S. Enteritidis (day 6)	*E. coli* O157 (day 6)	*C. jejuni* (day 11)
Red blood cells			
Haemoglobin	↓		↓
HCT			
MCV	↘		
MCH	↓↘	↓	
MCHC	↓	↓	↓
RDW	↓	↓	↑
HDW	↑	↑	
Platelets			
MPV	↓	↓	↓
White blood cells	↓↗		↓
Neutrophils	↗	↑	
Lymphocytes	↘	↓	
Monocytes	↓↗	↓	↓↗
Eosinophils			↓↘
Basophils	↓↗		↓
Large unstained cells (luc)	↓↗	↓	↓↗

↑ Significant increase in baseline level ($\beta > 0$) ↓ Significant decrease in baseline level ($\beta < 0$)
↗ Significant dose-dependent increase ($\gamma > 0$) ↘ Significant dose-dependent decrease ($\gamma < 0$)

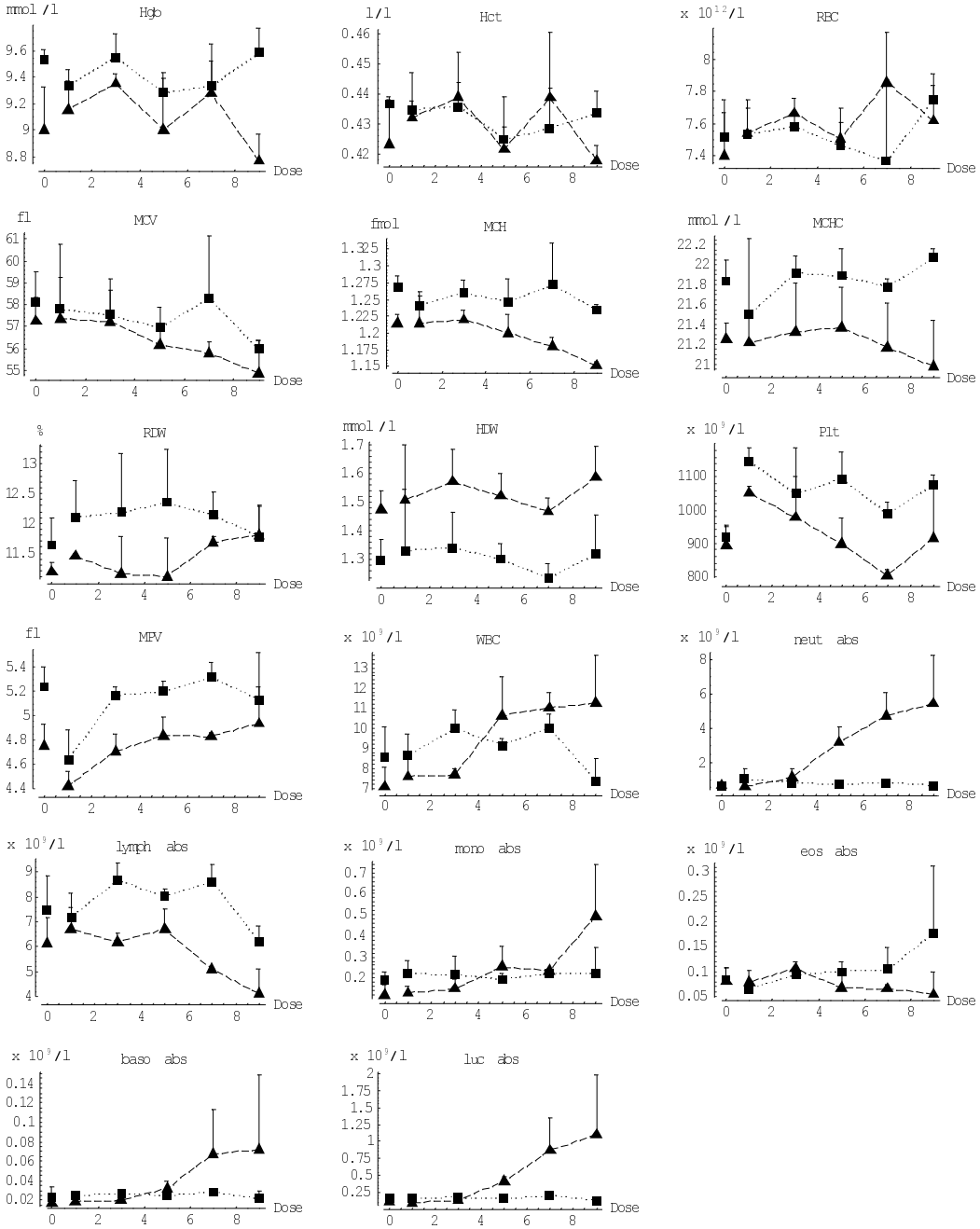

Figure 4. Haematological parameters in adult, male WU rats after oral exposure to S. *Enteritidis. Each symbol indicates the mean and standard deviation of 3 individual animals. Dose: log_{10} dose per animal, 0: control (WG5). ■ before exposure (day –1); ▲ after exposure (day 6).*

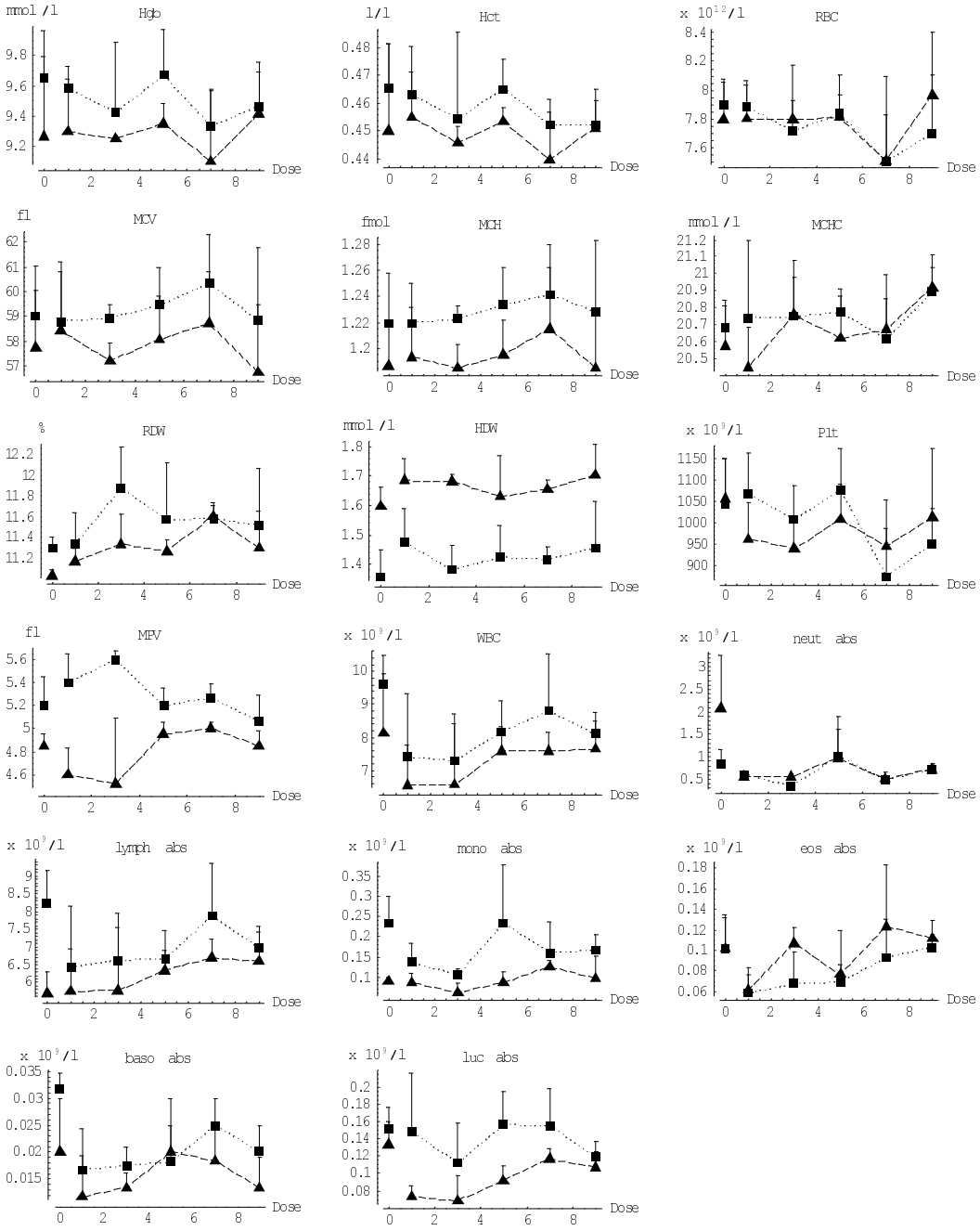

Figure 5. Haematological parameters in adult, male WU rats after oral exposure to E. coli *O157. Each symbol indicates the mean and standard deviation of 3 individual animals. Dose: log_{10} dose per animal, 0: control (WG5). ■ before exposure (day –1); ▲ after exposure (day 6).*

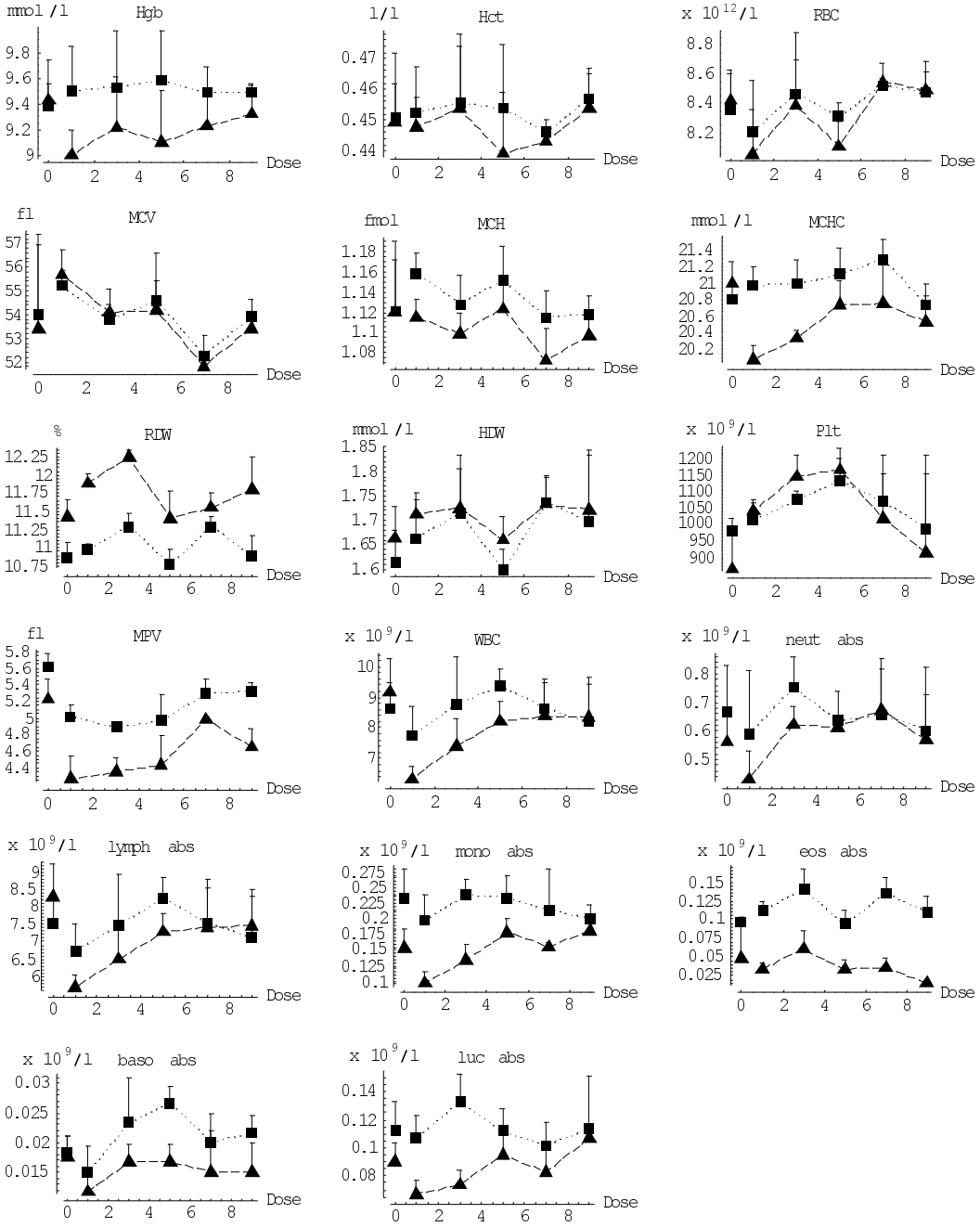

Figure 6. Haematological parameters in adult, male WU rats after oral exposure to C. jejuni*. Each symbol indicates the mean and standard deviation of 3 individual animals. Dose: log_{10} dose per animal, 0: control (WG5). ■ before exposure (day –1); ▲ after exposure (day 11).*

2.3.5 Pathology

In rats, infected with *E.Coli* O157 or *C. jejuni*, no gross or microscopical abnormalities were found. In rats, infected with *S.* Enteritidis, bronchopneumonia was detected in the highest dose group. Gross gastro-intestinal abnormalities were limited: little gastro-intestinal content in the highest dose *S.* Enteritidis dose group, and little gastric content in the second highest group, reflecting reduced food intake; enlarged Peyers' patches and mesenteric lymph nodes and thickening of the caecal wall in both groups.
Microscopically, in many animals (also in controls) isolated villi (< 1 per 100) distended by mononuclear cells (sometimes purely plasma cells) were seen, especially in the duodenum and jejunum. This was left aside when assessing the "itis"-score.
The histopathological findings of animals exposed to *S.* Enteritidis are summarised in Table 5. No abnormalities were seen in the duodenum and distal colon. In all but one animal in the two highest dose groups predominantly mononuclear inflammatory infiltration of the villi in the ileum was present, while the jejunum was generally normal. In one animal however the situation was the opposite. In order to illustrate these peculiarities, individual histopathological data are given in Appendix 2. Moderate to marked infiltration of the small intestinal mucosa ('villitis') was accompanied with a decreased villus-crypt ratio. Microgranulomas were seen mainly in Peyers' patches, in one animal associated with local peritonitis, and also in the lamina propria of animals in the two highest dose groups, as well as in one animal in the intermediate group. In the large intestine microgranulomas were limited to the GALT. Inflammation reached a higher grade of severity in the large intestine, especially in the caecum of the 3 highest dose groups. In the two highest groups diffuse flattening of superficial caecal epithelium, massive erosions and local ulcerations and decreased PAS-positivity were seen. In association almost diffusely, very marked, mixed inflammatory infiltration was present in the mucosa, near ulcers also in the submucosa, accompanied with marked oedema. Caecal lesions in the intermediate group locally reached equal severity, but were less extensive. Infiltration was also present in the proximal but not in the distal part of the colon.

Table 5. Histopathologic evaluation of enteritis (D: duodenum, J: jejunum, I: ileum, C: caecum, C1: proximal colon, C2: distal colon), of adult, male WU rats infected with different doses of S. *Enteritidis*

Dose (log_{10})	Severity class					
	D	J	I	C	C1	C2
9.4	-	++	+	+++	++	-
7.6	-	-	+	+++	++	-
5.5	-	-	+	+++	+	-
3.7	-	-	-	-	-	-
1.2	-	-	-	-	-	-
Control	-	-	-	-	-	-

Severity classes of enteritis: - no, + slight, ++ moderate, +++marked

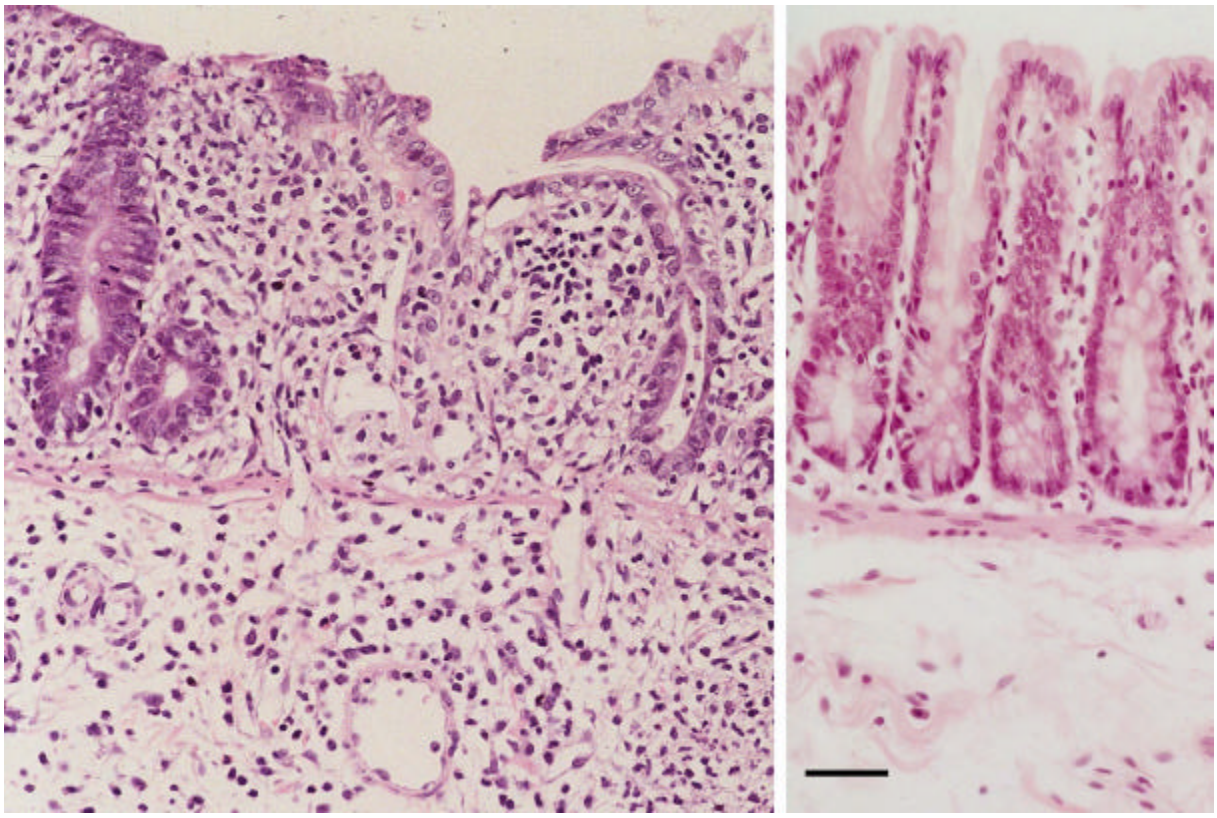

Figure 7. Left panel: destruction of crypts and diffuse epithelial flattening and erosion, massive mixed infiltration in (sub)mucosa of the caecum of a rat, infected with S. *Enteritidis (highest dose group). Right panel: normal control. Note high columnar epithelium, regular crypts and low cellularity of stroma. HE, bar is 40 mm.*

2.4 Conclusion

Exposure by intragastric gavage of adult, male WU rats to three enteropathogenic bacteria results in dose-dependent colonisation of the gastro-intestinal tract. *C. jejuni* was the most infectious of three species tested, with 2/3 animals infected at a dose of only 70 cfu per animal. *E. coli* O157 infected 2/3 animals at a dose of 5,700 cfu per animal, but 0/3 animals were infected at a dose of 50 cfu per animal. *S.* Enteritidis was least infectious, a dose of 330,000 cfu per animal but not lower doses infected 3/3 animals. For all bacteria, faecal counts increased with dose. For *C. jejuni*, peak values were obtained more rapidly at higher doses. *S.* Enteritidis was highly invasive, resulting in bacteraemia, pneumonia and reduced growth in the highest dose group. Macroscopically, limited gastro-intestinal abnormalities were observed only in the *S.* Enteritidis treated animals. There were only minor effects on body temperature and faecal moisture content. Microscopically, dose-dependent inflammation of the villi in the ileum, but not the duodenum, and granuloma in the Peyers' patches of the jejunum and ileum was demonstrated in animals exposed to *S.* Enteritidis. Severe, dose-dependent inflammation in the caecum and in the proximal colon was also observed in these animals. *E. coli* O157 and *C. jejuni* did not induce remarkable pathological changes. Experimental conditions (probably repeated blood sampling) induced anaemia in all animals, including control groups. In the highest dose groups of *S.* Enteritidis, anaemia was significantly more pronounced than in other groups. White blood cells (notably neutrophils) increased markedly in animals exposed to *S.* Enteritidis, with a highly significant dose-response relation.

3. Reproducibility of infection with *C. jejuni*

3.1 Introduction

In the previous experiments, *C. jejuni* was found to be the most infectious of three species tested. This chapter describes a repeat experiment involving more animals that were exposed to inocula in a smaller dose-range, and with smaller dilution steps. The objectives were to obtain more precise information on the dose-response curve, and to test the reproducibility of the rat model.

3.2 Materials and methods[2]

The animals, bacterial strains, experimental design and all analytical protocols were the same as described in 2.2.4, with the following exceptions. The experiment was divided into two cohorts, each dose group consisted of 4 animals per cohort. A 48 hour culture of *C. jejuni* B258 was washed by filtration as described in 2.2.3 and diluted 10^5 times. This suspension was then twofold diluted from 1:2 to 1:16. An overnight culture of *E. coli* WG5 was diluted 10^6 times. Directly before administration to the animals, 6 ml of each bacterial suspension was mixed with 6 ml of a solution of 6% (w/v) $NaHCO_3$.

3.3 Results

3.3.1 Inoculum cultures

Table 6 shows the plate counts of different dilutions of the inoculum culture. For convenience in later sections, each dose group is assigned a letter code. The intended range of 10-1000 cfu per animal was obtained in the first cohort, but in the second cohort the doses were about 10-fold higher.

Table 6. Microbiological analysis (colony count per ml) of C. jejuni *in inoculum cultures*

Dose group	cohort 1	cohort 2
Highest	530	6200
Second highest	240	3000
Intermediate	140	1300
Second lowest	68	550
Lowest	16	220
Control (WG5)	500	280

3.3.2 Clinical and macroscopic observations

No clinical signs of infection or disease were observed. At necropsy, no abnormalities were detected.

[2] This experiment is registered as AAP/199800792

3.3.3 Microbiology

Only one of 40 animals, infected with *C. jejuni* in doses between 16 and 6200 per animal was infected as indicated by faecal excretion (Table 7). In this animal, faecal counts varied between 10^2 and 10^5 cfu/g.

Table 7. Faecal excretion of C. jejuni *by adult, male W rats after oral exposure to different doses*

Dose group	cohort 1	cohort 2
Highest	0/4	0/4
Second highest	1/4	0/4
Intermediate	0/4	0/4
Second lowest	0/4	0/4
Lowest	0/4	0/4
Control (WG5)	0/4	0/4

In view of these results, it was decided not to report haematological data. Histopathological examinations were not performed.

3.4 Conclusion

The reproducibility of the rat model with *C. jejuni* as a test organism is very poor. No obvious explanation was found when reviewing the experimental conditions: the rats were obtained from the same breeding colony, and were colonised with the same CRF flora. There were minor differences in the period of fasting (24 h in this experiment vs. 18 h in the previous) but this would be expected to increase the animals' susceptibility instead of decreasing it. The inoculum was prepared in the same way, and was viable as demonstrated by the plate counts after exposure of the rats. Two possible sources of experimental variation can be suggested. One is the overall sensitivity of *C. jejuni* to environmental conditions, particularly to oxygen. This may influence the infectivity of the bacteria in subtle way that is not detected by plate counts. More detailed tests of the viability of the inoculum need to be developed to evaluate this hypothesis. A second possibility is variation in the composition of the animals' diet. At the RIVM, rats are fed a conventional diet, the composition of which is known to vary with the (seasonal) availability of ingredients. Loesberg (1989) demonstrated that even minor changes in food pellets that are not normally reported to scientists, may induce significant alterations in the immune system of rodents and thus could also influence the rats' susceptibility to bacterial infection. In that case, it would be necessary to change to a synthetic diet.

4. Reproducibility of infection with *S.* Enteritidis

4.1 Introduction

The poor and presently unexplained reproducibility of infection with *C. jejuni* (see Chapter 3) prompted the question if this problem would also occur with *S.* Enteritidis, which is a more robust organism. Therefore, a second series of experiments with this species was planned, again spanning a wide dose-range from 10^3 to 10^{10} cfu per animal.

4.2 Materials and methods[3]

4.2.1 Experimental design

The animals, bacterial strains, experimental design and all analytical protocols were the same as described in 2.2.4, with the following exceptions. Five groups consisting of five rats were given either *E. coli* WG5 or different dilutions of a culture of *S.* Enteritidis. The remaining parts of the undiluted bacterial suspensions were heat-killed at 80°C for 10 min and conserved by freezing at –20 °C for the DTH reaction (see 4.2.2). Histopathological examination was confined to the caecum.

4.2.2 Delayed type hypersensitivity-reaction (DTH)

Five days after oral infection with *S.* Enteritidis the thickness of both ears of each animal in each group was assessed in duplicate using an engineering micrometer (Mitutoyo 193-10, Veenendaal, The Netherlands). For this purpose, the animals were anaesthetised by intraperitoneal injection of 100 µl of a KRA solution (a cocktail consisting of 7 ml ketalar (50 mg/ml, Parke Davis, Spain), 3 ml rompun (20 mg/ml, Bayer, Leverkusen, FRG), and 1 ml of atropin (1mg/ml, OPG, Utrecht, The Netherlands). Directly after the ear measurements 25 µl of the heat-killed suspension of *S.* Enteritidis (approximately 5 x 10^8 cfu/ml) was subcutaneously injected into the pinnae of each ear of each rat (also in the control WG5 group!). The increase in ear-thickness was assessed 24 hrs after challenge under ether anaesthesia. The difference between ear-thickness prior to and 24 hrs after injection was calculated and reflects the DTH response, a valid parameter for T cell dependent (in vivo) immunity to *S.* Enteritidis. The swelling in control animals reflect the background swelling response induced by the ear injection of *S.* Enteritidis itself.

4.3 Results

4.3.1 Inoculum cultures

Table 8 shows the plate counts of different dilutions of the inoculum culture, again assigned a letter code for each group. Note that all doses were approximately 10-fold lower than in the previous experiment (Table 1). By mistake, the inoculum of the low dose group was plated on the wrong medium, hence the dose is not known directly. In the table, an estimated range is given on the basis of the counts in the higher dose groups and the dilution ratio.

[3] This experiment was registered as AAP/199900105

Table 8. Microbiological analysis (colony count per ml, log_{10} in brackets) of inoculum cultures

Dose group	*S.* Enteritidis
Highest	2.6×10^8 (8.4)
Second highest	3.1×10^6 (6.5)
Intermediate	4.9×10^4 (4.7)
Low[#]	3×10^2 (2.5)
Control (WG5)	2.7×10^8 (8.4)

[#] Extrapolated from higher dose groups, see text

4.3.2 Clinical observations

The animals infected with the two highest doses showed severe illness from day 4 after infection onward. The animals were skinny, weak, cold, had a ruffled fur, red crusts around eyes and nose tips, and increased amounts of nasal fluid. Severe systemic illness from day 3 onwards occurred in 2/5 rats in the highest dose-group, these were sacrificed and necropsied at day 5, the other rats on day 6 as planned. In the highest dose group a loss of mean body weight from day 2 onwards was noted (see Figure 8).

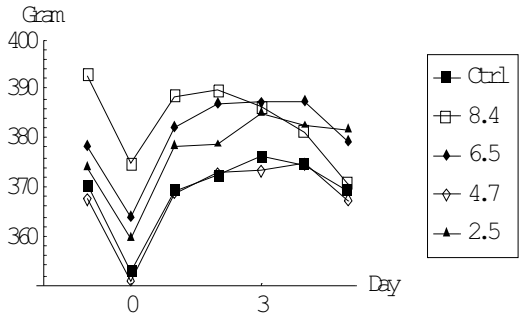

Figure 8. Body weights of adult, male WU rats after oral exposure to S. Enteritidis. Each symbol indicates the mean of 3 individual animals. Legend: log_{10} dose per animal, Ctrl: control (WG5).

The caecum weights in all animals were less than 2% of the total body weight, indicating no major abnormality in the rat's intestinal microflora. Compared to the control group, no significant difference in caecum to body weight ratio was found in all groups infected with *S.* Enteritidis except the second highest dose group (see Figure 9).

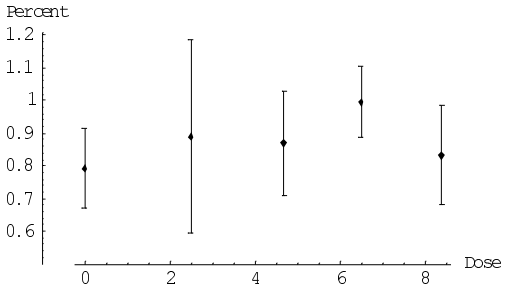

Figure 9. Caecum to body weight ratio of adult, male WU rats after oral exposure to S. *Enteritidis. Each symbol indicates the mean and standard deviation of five individuals (three for dose 8.4). Dose: log_{10} dose per animal, 0: control (WG5).*

There was a dose-dependent decrease of body temperature of the infected animals, for measurements taken in the afternoon. Because of relatively high inter-individual variability, the difference only reached statistical significance for the second highest dose group (two-sided t-test of exposed animals versus controls, see Figure 10) but not for the highest dose group, even though the mean temperature was considerably lower. Temperature measurements taken during the morning did not show any dose-dependent differences. This may be related to the fact that rats are night-feeders, and can maintain their body temperature by physical activity despite the effect of infection. During daytime, the animals sleep and a decrease in body temperatures is not compensated by physical activity.

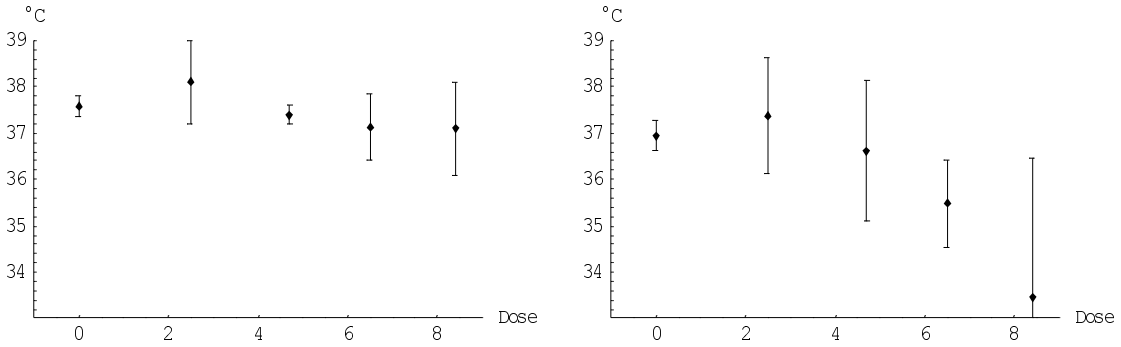

Figure 10. Body temperature (afternoon measurements) of adult, male WU rats after oral exposure to S. *Enteritidis. Each symbol indicates the mean and standard deviation of five individuals. Dose: log_{10} dose per animal, 0: control (WG5). Left panel: day –1; right panel: day 5.*

In the two highest dose groups, significant rises in faecal moisture content were observed over 7 days (day –1 to day 5, see Figure 11).

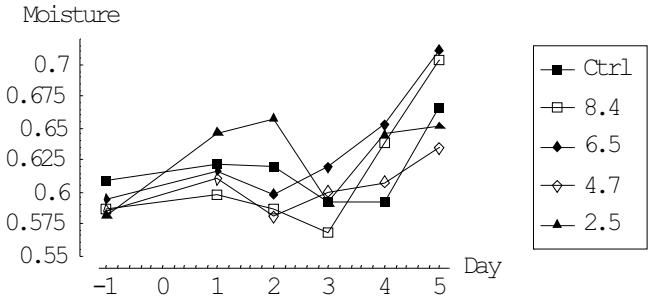

Figure 11. Moisture content of the faeces of adult, male WU rats after oral exposure to S. Enteritidis. Each symbol indicates the mean of five individual animals. Legend: log_{10} dose per animal, Ctrl: control (WG5).

4.3.3 Microbiology

Figure 12 shows the faecal excretion of pathogens in relation to dose and time after inoculation. As in the previous experiment, exposure to *S.* Enteritidis resulted in a sharp increase of faecal counts, followed by a transient decrease and subsequent increase. Even though the doses were tenfold lower than in the previous experiment, the excretion patterns were very similar. No *E. coli* WG5 was detected in any of the faecal samples, blood or lymph nodes.

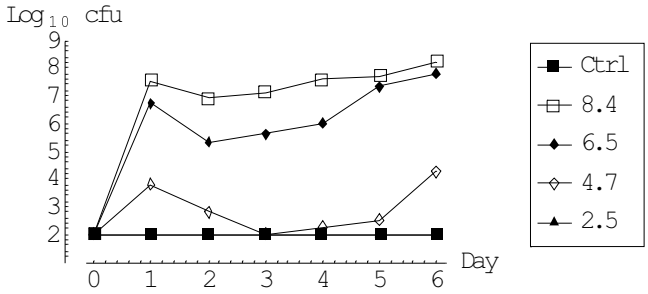

Figure 12. Colony counts in the faeces of adult, male WU rats after oral exposure to S. *Enteritidis. Each symbol indicates the mean of 5 individual animals. Legend: log_{10} dose per animal, Ctrl: control (WG5).*

If again the criterion for infection is defined as a faecal count > 1000/g on at least two days, the dose-response data as shown in Table 9 are obtained. Infection was again observed in the three highest dose groups, hence down to a dose of 5 x 10^4 cfu per animal.

Table 9. Infection (defined by faecal excretion) of adult, male WU rats after oral exposure to S. Enteritidis

Dose group	Rats infected/exposed
Highest	5/5
Second highest	5/5
Intermediate	5/5
Low	0/5

The counts in mesenteric lymph nodes and spleen are shown in Table 10. Infection occurred in all dose-groups, also in the lowest group, where faecal excretion was not detected. The counts in mesenteric lymph nodes in lower dose groups were inaccurate because of strong interference by contaminating background flora. The number of animals with positive mesenteric lymph nodes increased with dose. Also, there was a tendency of increasing counts in positive lymph nodes with increasing dose. This pattern was very obvious for counts in the spleen.

Table 10. S. *Enteritidis (cfu per gram) in mesenteric lymph nodes and spleen of adult, male WU rats after oral exposure to different doses*

Dose group	Mesenteric lymph nodes	Spleen
Highest	2.5 x 10^5 (5)	9.6 x 10^3 (5)
Second highest	1.4 x 10^5 (5)	1.7 x 10^4 (5)
Intermediate	✱ (3)	1.1 x 10^4 (5)
Low	✱ (1)	4.4 x 10^3 (2)

Each data point indicates the mean of positive animals. Shown in brackets is the number of positive animals out of five.
Asterisk (✱): interference by background flora

4.3.4 Delayed-type hypersensitivity reaction (DTH)

In all infected animals a significant specific delayed type hypersensitivity response was detectable as compared to the background swelling response induced by *Salmonella* injections in the ears of control rats (see Figure 13). This indicates a significant and dose-related systemic T cell dependent immune response to the *S.* Enteritidis. Even in the lowest dose group a significant response was detectable which is consistent with the presence of *S.* Enteritidis in the spleen.

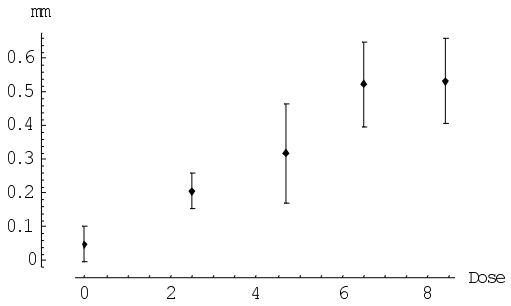

Figure 13. DTH reaction in adult, male WU rats after oral exposure to S. *Enteritidis.*
Each symbol indicates the mean and standard deviation of 5 individual animals (3 in the VH group) .
Dose: log_{10} dose per animal, 0: control (WG5).

4.3.5 Haematology

Detailed information on the results of haematological analysis is given in Figure 14. Table 11 shows a summary of significant changes in the blood parameters.

Table 11. Changes in haematological parameters in adult, male WU rats after oral exposure to S. *Enteritidis*

Red blood cells	↑
Haemoglobin	
HCT	↑
MCV	↓
MCH	↓↘
MCHC	↓
RDW	
HDW	↑
Platelets	
MPV	↗
White blood cells	↗
Neutrophils	↑↗
Lymphocytes	↓
Monocytes	↗
Eosinophils	↘
Basophils	↗
Large unstained cells	↗

↑ Significant increase in baseline level ($\beta > 0$)
↓ Significant decrease in baseline level ($\beta > 0$)
↗ Significant dose-dependent increase ($\gamma > 0$)
↘ Significant dose-dependent decrease ($\gamma < 0$)

Again, there was a significant decrease in the baseline level of some haematological parameters (MCV, MCH, MCHC, and lymphocytes). Note that in the previous experiment more parameters were decreased as compared to baseline (Hgb, RDW, MPV, WBC, monocytes, basophils, LUCs), whereas in this experiment there was also an increase in the baseline level of some parameters (RBC, HCT and neutrophils). Thus, anaemia induced by experimental conditions was less pronounced than in the previous experiment. In this experiment, blood was only sampled at day –1, but not at day 3. The increase in neutrophils may have been induced by inoculation of heat-killed *S.* Enteritidis for the DTH reaction. As in the previous experiment, a dose-dependent increase in the number of phagocytic cells involved in inflammatory reactions (neutrophils, monocytes) was observed.

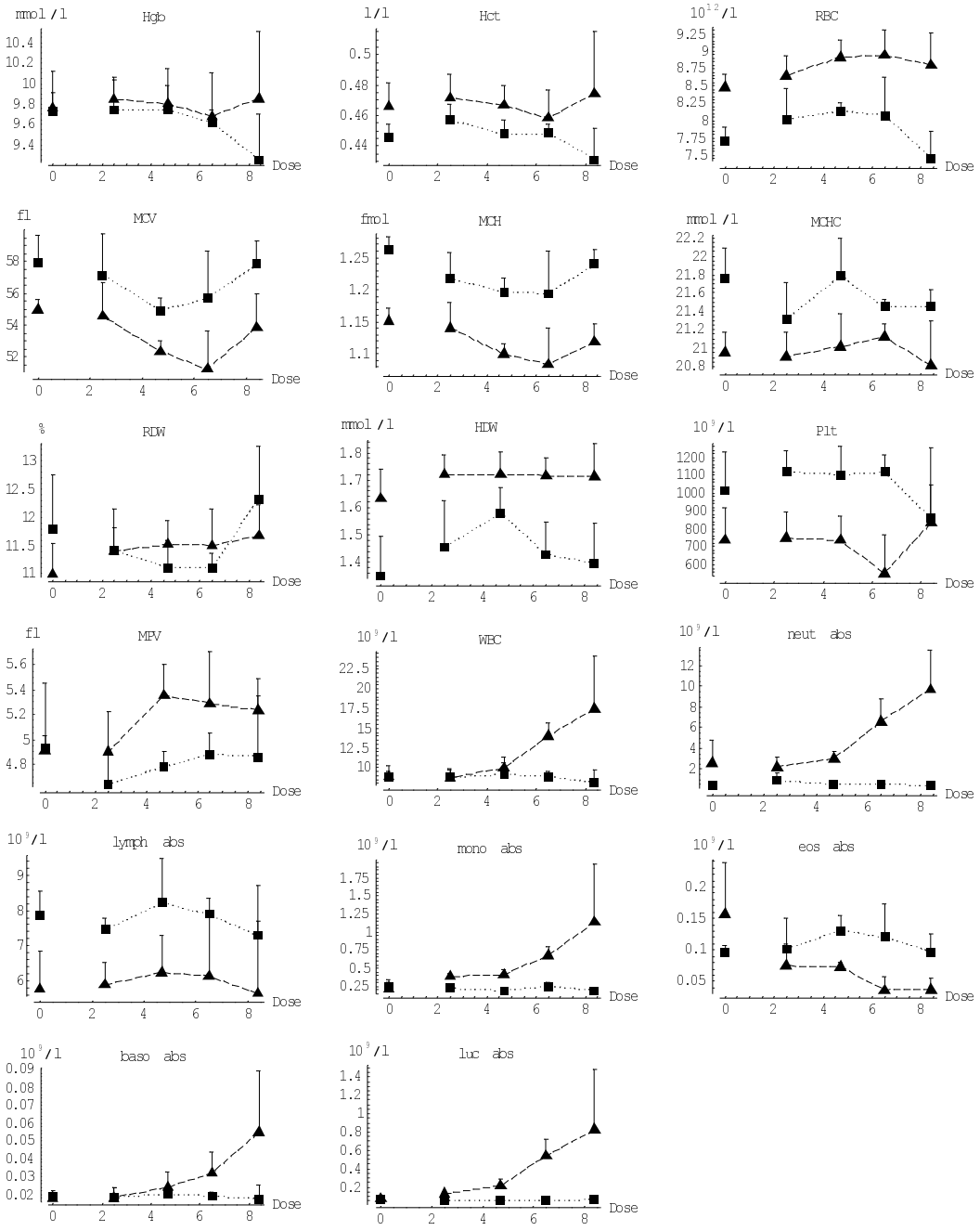

Figure 14. Haematological parameters in adult, male WU rats after oral exposure to S. *Enteritidis. Each symbol indicates the mean and standard deviation of 5 individual animals. Dose: log_{10} dose per animal, 0: control (WG5). ■ before exposure (day –1); ▲ after exposure (day 6).*

4.3.6 Pathology

A summary of the macroscopic findings is given in Table 12. In general, the effects were more pronounced than in the previous experiment. There was dose-related systemic illness, notably pneumonia and hydrothorax. Also, there were limited, dose-related gastro-intestinal abnormalities: little gastro-intestinal content reflecting reduced food intake, abnormal, crumbly gastro-intestinal content (not seen in previous studies) and opacity of the caecal wall. Peyers' patches in the ileum and the mesenteric lymph nodes were enlarged in the two highest dose groups. The two rats that were killed on day 5 were the only ones in their group without obvious typhlitis (mainly crypt hyperplasia), but caecal GALT showed microgranulomas in both.
Histopathological lesions in the caecum (App. 3) were comparable to those seen in the previous study (2.3.5), but more variation in the severity of mucosal lesions was observed in the intermediate dose group. In caeca, abnormal mucosa predominated and submucosal (sometimes transmural) inflammatory infiltration was marked to severe.

Table 12. Macroscopic findings in young, adult WU rats, exposed to different doses of S. *Enteritidis*

Dose group	Pneumonia	Hydrothorax	Opacity caecum	Little GI contents	Abnormal GI contents
Highest	3/5	3/5	4/5	4/5	3/5
Second highest	0/5	2/5	1/5	4/5	4/5
Intermediate	0/5	1/5	0/5	2/5	1/5
Low	0/5	0/5	0/5	2/5	0/5
Ctrl (WG5)	0/5	0/5	0/5	0/5	0/5

4.3.7 Conclusion

The dose-response relationship of exposure by intragastric gavage of adult, male WU rats to *S.* Enteritidis appears to be well reproducible. As in the previous experiment, the animals were shedding the bacteria in their faeces after exposure to intermediate to high doses (> 10^4 cfu/animal). At a lower dose (300 cfu/animal), no faecal excretion was detected within the 6-day period of observation but salmonellae could be isolated from the spleen and mesenteric lymph nodes. Furthermore, already at this dose there was a positive DTH-reaction. These findings underline the fact that *S.* Enteritidis is highly invasive in rodents and that the intestinal tract is not the major site of multiplication.
Pathological results confirm observations in a previous study (Garssen *et al*., 1998), which indicated that the gastro-intestinal tract (although portal of entry) shows relatively little abnormalities in animals, that succumb after oral inoculation with very high doses of *S.* Enteritidis. On the other hand, systemic illness was severe, as demonstrated by pneumonia and hydrothorax.

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Appendix 1 Mailing list

1. Director-General RIVM
2. S. Leach, CAMR, Porton Down, UK
3. V. Leclerc, AFSSA, Maison Alfort, France
4. J. Huis in 't Veld, Utrecht University
5. F. van Knapen, Utrecht University
6. R. de Waard, Utrecht University
7. W. Gaastra, Utrecht University
8. B. van Middelaar, Utrecht Medical Centre
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26. D. Elberts
27. H. van Loveren
28. P. Steerenberg
29. M. van Dijk
30. W. de Jong
31. C. Moolenbeek
32. P. Roholl
33. Y. Wallbrink
34. P. Steerenberg
35. P. Beekhof
36. E. Delfgou-van Asch
37. W. Ritmeester
38. R. de Jonge
39. W. van Leeuwen
40. W. Jansen

41-48. Authors

49. SBD/Voorlichting & Public Relations
50. Bureau Rapportenregistratie
51. Bibliotheek RIVM

52-63. Bureau Rapportenbeheer

64-70. Reserve

Appendix 2 Individual histopathological data, *S.* Enteritidis, experiment AAP/199800164

dose (log_{10})	**animal nr.**	**duodenum** villitis	**jejunum** villitis	**ileum** villitis	**caecum** infiltration	**colon 1** infiltration	**colon 2** infiltration
Control	1	-	-	<1	1	1	1
	2	<1	-	<1	1	1	1
	3	<1	<1	-	1	1	1
9.4	4	1	2	3	5	3	1
	5	1	4	-	5	3	1
	6	1?*	1?*	3	5	3	1
7.6	7	<1	1	2	5	3	1
	8	-	<1	2	5	3	1
	9	<1	1	3	5	3	1
5.5	10	?*	?*	?*	?*	?*	?*
	11	<1	1	2	4 à 5	2	1
	12	<1	<1	<1	4	1 à 2	1
3.7	13	-	<1	<1	1	1	1
	14	-	<1	<1	1	1	1
	15	-	<1	1	1	1	1
1.2	16	<1	<1	-	1	1	1
	17	-	<1	1	1	1	1
	18	<1	-	-	1	1	1

Legend: See Appendix 3
The villus-crypt ratio in the control animals was estimated at duodenum 4 à 5: 1; jejunum ± 3: 1, ileum ± 2: 1.
*autolytic

Appendix 3 Individual histopathological data, *S.* Enteritidis, experiment AAP/199900105

Dose (log^{10})	animal nr.	caecum	
		infiltration	GALT-granuloma
Control	1	1	-
	2	1	-
	3	1	-
	4	1	-
	5	1	-
8.4	6	1	+
	7	5	++
	8	5	±
	9	1.5	+
	10	5	+
6.5	11	4	+
	12	5	no sample
	13	5	++
	14	5	++
	15	5	-
4.7	16	3	-
	17	1	-
	18	1	no sample
	19	3	no sample
	20	4	±
2.5	21	1	-
	22	1	-?
	23	1.5	-
	24	1	no sample
	25	1	-

Infiltration score: -, no abnormality; 1, minimal; 2, slight; 3, moderate; 4, marked; 5, severe.
granuloma score: ++, 50% or more of GALT occupied by granuloma

Abbreviations
infiltration oedema /inflammatory infiltration in lamina propria of large intestine (caecum and colon)
GALT-granuloma granuloma in gut associated lymphoid tissue