*Epidemiol. Infect.* (1991), **107**, 619–626
*Printed in Great Britain*



# Study of colonization resistance for Enterobacteriaceae in man by experimental contamination and biotyping as well as the possible role of antibodies in the clearance of these bacteria from the intestines

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(*Accepted 20 May 1991*)

SUMMARY

The colonization resistance (CR) of the digestive tract was determined in 10 healthy volunteers by oral contamination with a neomycin resistant *Escherichia coli* (NR-*E. coli*) strain and measurement of the faecal concentration of this strain during 14 days after the contamination. This 'gold standard' was compared with another parameter of CR; the determination of the mean number of different biotypes of Enterobacteriaceae isolated from four faecal samples per volunteer. Both measures are significantly correlated ($P < 0{\cdot}01$). The NR-*E. coli* strain could be cultured from faecal samples of 4/10 volunteers as long as 300 days after contamination. Serum antibody titres against endogenous *E. coli* strains and the NR-*E. coli* strain used for experimental oral contamination were measured by an indirect immunofluorescence (IIF) assay. The assay was read by a video camera connected to an image processing system. The 95% confidence limits of antibody titres ($\log^2$) against endogenous *E. coli* strains ranged between $< 3$ and 7·1 for IgA, between $< 3$ and 8·7 for IgG and between $< 3$ and 7·4 for IgM. Antibody titres against the NR-*E. coli* 4 strain were within this (normal) range. The serum antibody titres against the NR-*E. coli* strain increased slowly after oral contamination, especially IgG and IgM. Little increase in IgA titres could be observed. An increase of serum antibody titres did not correlate with the elimination of the oral contaminant from the intestines. Therefore, we conclude that the CR is not IgG nor IgM antibody mediated.

## INTRODUCTION

In patients with decreased immunological defence mechanisms (colonization resistance) CR is important. CR provides protection by the indigenous microflora with respect to colonization of the digestive tract by potentially pathogenic microorganisms. The quality of CR and hence its protective capacity may vary between individuals of the same species [1].

Study of the quality of CR can be used as a functional parameter of the faecal indigenous microflora in studies concerning the host response to the indigenous faecal microflora and its impact on the functioning of the immune system. If

https://doi.org/10.1017/S0950268800049311 Published online by Cambridge University Press

bacteria play a role in the pathogenesis of autoimmune phenomena as found in animal studies [2], this type of research may be valuable for patients with a systemic autoimmune disease. We have studied the relationship between CR and antibodies directed against human indigenous faecal bacteria in patients with systemic autoimmune disease and in healthy persons, bearing in mind the likely greater susceptibility of autoimmune patients to infection.

In mice CR has been defined as the $\log^{10}$ of the oral bacterial dose which is followed by a persistent 'take' in 50% of the contaminated animals [3]. For an individual mouse the CR can be directly expressed as the $\log^{10}$ concentration of a specific potentially pathogenic bacterial species found in the faeces two weeks after contamination. Measurement of CR can also be accomplished by quantitative biotyping of one or more Enterobacteriaceae strains isolated from faeces collected at three of four daily intervals. This was considered a useful method because of the finding that, after oral administration of a single dose of an *Escherichia coli* strain to mice, the population density of these bacteria in the intestinal tract (faeces) varied inversely with CR. The mean concentration of the typed bacteria was determined using a time related concentration curve [4].

In man the two methods for determining CR, i.e. oral contamination and biotyping, have never been used simultaneously. Our first step was to verify the value of biotyping in man following oral contamination.

Ten healthy volunteers were given orally a NR-*E. coli* strain. Thereafter the mean faecal concentration of this strain was assessed from day 1 to day 14. These concentrations were compared with the mean number of different biotypes of Enterobacteriaceae in the faeces in four faecal samples obtained from each volunteer within 1 week prior to the oral contamination [6].

We also determined the isotypes and titres of serum antibodies directed against the endogenous (faecal) *E. coli* strains and against the NR-*E. coli* strain. Where individuals have high CRs the faecal concentration of their strains of Enterobacteriaceae would be likely to be less than in persons with a lower CR. The intestinal (faecal) concentration of Enterobacteriaceae is clinically important because high numbers are correlated with translocation to the lymphatic organs and the liver [5]. Serum antibodies, therefore, could be lower in individuals with a high CR than in a person with a low CR of his digestive tract. It is not clear whether the existence or any rise in serum antibody titres against endogenous Gram-negatives is merely the consequence of this translocation or whether it plays a role in the CR; i.e. clearance of these bacteria from the intestinal tract. To study the possible relationship of a humoral immune response to this clearance, we determined the serum antibody titres and isotypes against the NR-*E. coli* strain.

## MATERIALS AND METHODS

### *Volunteers and sampling*

Ten healthy volunteers, seven males and three females, aged 22–44, entered this study after having given written informed consent. The experiment was approved of by the Medical Ethical Committee of the University Hospital Groningen.

Before oral contamination, four faecal samples were collected per volunteer within 1 week, as well as one blood sample. After oral contamination with an *E.*

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*coli* strain, serum samples were collected on day 4, 14 and 128 and faecal samples were collected daily, whenever faeces were produced. After 3 months the frequency of faecal sampling diminished to once per month. In the period of 1 month before until 3 months after the oral contamination, no volunteer had suffered or did suffer from a gastrointestinal disease or had taken antibiotics.

*Oral contaminating strain*

The *E. coli* strain used for oral contamination was identified with the API-20 E system (Analytab Products Inc., Montalieu Vercieu, France). The biotype of the strain was 1144512. The strain was resistant to neomycin (minimal inhibitory concentration > 250 mg/l). The strain will be referred to as NR-*E. coli*.

For oral contamination the volunteers ingested 10 ml chocolate milk to which 1 ml of an overnight culture at 37 °C (approximately $10^9$ bacteria/ml) of the NR-*E. coli* strain had been added.

*Determination of the faecal concentration of the NR-*E. coli *strain*

Faeces were suspended 1:9 (w/v) in Brain Heart Infusion broth (BHI, Oxoid, Basingstoke, UK) and subsequently diluted 1:9 (v/v) in BHI. After overnight incubation at 37 °C the suspensions were subcultured on MacConkey agar (Merck, Darmstadt, FRG) to which neomycin had been added (250 mg/l). The concentrations were determined by standard dilution methods for both the NR-*E. coli* strain and the endogenous strains of Enterobacteriaceae.

*Measurement of CR by comprehensive biotyping of Enterobacteriaceae*

CR was defined as the reciprocal value of the mean number of different biotypes of Enterobacteriaceae isolated from four faecal samples obtained within 1–2 weeks [6]. Details of the biotyping technique have been described previously [7]. The strains were stored at −20 °C until use for specific antibody titrations.

*Measurement of CR by oral contamination*

CR can be expressed directly as the $\log^{10}$ concentration of a specific potentially pathogenic bacterial species found in the faeces 2 weeks after contamination. Because of the normal fluctuations in the faecal concentration of Enterobacteriaceae we assessed the mean faecal concentration of this strain daily from day 1 until day 14 after oral contamination instead of the faecal concentration on day 14 only.

*Serum*

Blood samples were centrifuged in a Beckman centrifuge type TJ-6 (Palo Alto, California, USA) for 10 min at 1420 g 1 h after venepuncture. The supernatant was then placed in a water bath of 56 °C for 30 min to inactivate complement. Thereafter, the serum was stored at −20 °C in aliquots of 0·2 ml.

*Measurement of serum antibody titres against endogenous and oral contaminating* E. coli *strains by IIF read by a videocamera and an image processing system*

Details of the technique have been described [8]. Briefly, overnight cultures of each of the *E. coli* strains were washed and suspended in demineralized water with 0·5% Tween 80 (Merck), placed separately on 12-well microscope slides

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Table 1. *Concentration ($log^{10}/g$) of the NR-*E. coli *strain in the faeces of 10 healthy volunteers during 14 days after ingestion*

| | Volunteer | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | K |
| Day | | | | | | | | | | |
| 1 | 6·5 | 4·5 | 2 | 6 | 6 | 4 | 4·5 | 3 | —* | 4 |
| 2 | — | 4 | 3·5 | 5 | 4·5 | 4 | 5 | 3 | 5 | 4 |
| 3 | 5 | 2 | 4·5 | 4 | — | 3·5 | 5 | 2 | 3 | 4 |
| 4 | 5·5 | 1 | 3 | — | 4 | 3 | 5 | 4 | 5 | 4 |
| 5 | 3·5 | 0 | 4·5 | 5 | 1 | — | — | 2·5 | — | — |
| 6 | 2·5 | 0 | 2·5 | 3·5 | 4 | 1 | — | 2·5 | 4·5 | — |
| 7 | — | 0 | 4 | 3 | 4·5 | 2 | 5 | 4·5 | 5·5 | 4 |
| 8 | — | 0 | 4 | 2 | — | 2·5 | 4 | 6·5 | — | 4·5 |
| 9 | 5 | 0 | 1 | 5·5 | 4·5 | 2·5 | — | 4 | — | 3 |
| 10 | 5 | — | 3 | 4 | — | 0 | 4 | 5 | 6 | 3 |
| 11 | — | — | 4·5 | 4 | 2·5 | — | 5 | 4·5 | — | 4·5 |
| 12 | 5 | — | 3·5 | 4·5 | 2 | 1 | 2 | 2·5 | 3·5 | 3·5 |
| 13 | 3·5 | — | 3 | 5 | 3 | 0 | — | 3·5 | 5·5 | 2·5 |
| 14 | 3 | — | 3 | 4·5 | 3·5 | 0 | 5 | 3·5 | 5·5 | 3 |
| 128 | 3 | — | 3 | 1 | 4·5 | — | 4·5 | — | 3 | 1 |

* not determined.

(Immunocor, Limoges Cedex, F) and fixed in acetone. After washing with PBS, dilutions of serum (from the person from whom the cultures were obtained) were added to the wells and the slides incubated at room temperature in a moist chamber for 45 min. Antibodies were studied only to strains isolated from each individuals own faeces. After washing with PBS, the slides were incubated with fluorescein isothiocyanate (FITC) conjugated goat-anti-human $F(ab')_2$ IgA, IgM and IgG (Kallestad, Texas, USA) for 1 h in a moist chamber at room temperature. After mounting slides stored at 4 °C until examination (within 24 h). For every strain duplicate preparations were made.

The slides were read by an image processing system consisting of an Olympus BH2 microscope (Olympus Optical Co., LTD., Tokyo, Japan) equipped with a Fairchild CCD 5000/1 camera (Fairchild Weston Systems Inc. Sunnyvale California USA) connected to an AT computer with a Matrox MVP/AT(/NT) image processing board. Software for reading and evaluating data was developed in our laboratory [8, 9]. The software combines measuring of micromorphology of the bacteria with scoring of the IIF of each the 100 bacteria measured per microscope well.

## RESULTS

The strains of Enterobacteriaceae isolated from the faecal samples of the volunteers collected in the week before the oral contamination did not grow on the MacConkey agar to which neomycin had been added. It was thus concluded that they were sensitive to neomycin in contrast to the strain used for oral experimental contamination. The oral intake of the chocolate milk with the NR-*E. coli* strain was well tolerated as none of the volunteers suffered from any clinical symptoms. The strain was not cleared from the intestines within 14 days except in one case.

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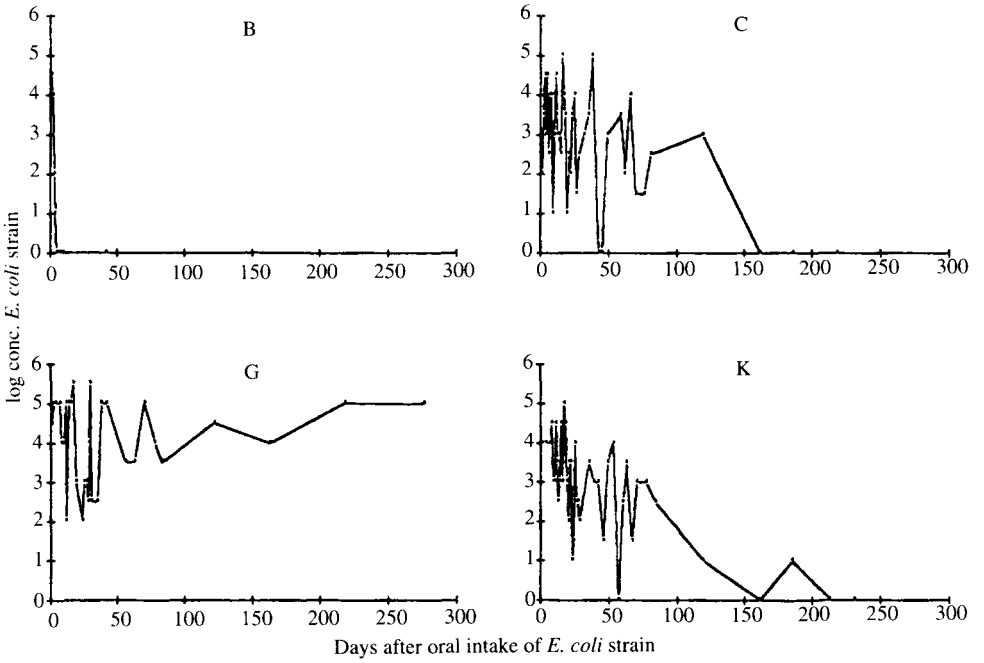


Fig. 1. The clearance of NR-*E. coli* in four subjects; volunteer B eliminated the strain in 4 days, volunteers C and K in 160 and 210 days respectively, and volunteer G remained persistently colonized by the strain during the entire 300 days additional study period.

The results of cultures taken during the first 14 days are shown in Table 1 and those for the extended period of 1 year after the oral contamination are shown in Fig. 1. These data show that after 1 year four volunteers were still colonized with the NR-*E. coli* strain.

The mean number of different biotypes isolated from the faecal samples collected prior to the oral contamination ranged between 1·25 and 2·25 between the volunteers. The mean concentration of the NR-*E. coli* strain measured from day 1 to 14 correlated significantly with the mean number of different biotypes of Enterobacteriaceae isolated from the four faecal samples of the volunteers collected before the oral contamination ($r = 0{\cdot}80$, $P < 0{\cdot}01$, Fig. 2). The mean concentration of Enterobacteriaceae in the faecal samples before the oral contamination ranged between 2·4 and 4·6 ($\log^{10}$/g faeces). No correlation was found between the mean concentration of Enterobacteriaceae in the faecal samples before the oral contamination and the mean concentration of the oral contaminating strain measured from day 1 to 14 ($r = -0{\cdot}01$).

The serum antibody titres and their isotypes against the NR-*E. coli* strain before and after the oral contamination as well as the serum antibody titres against the endogenous strains of Enterobacteriaceae isolated from the faeces before the oral contamination are shown in Table 2. If production of serum antibodies is dependent on the concentration of the ingested strain, their colonization of the mucous membrane and their translocation, one would expect the highest average antibody titres in those persons with the lowest CR. This could

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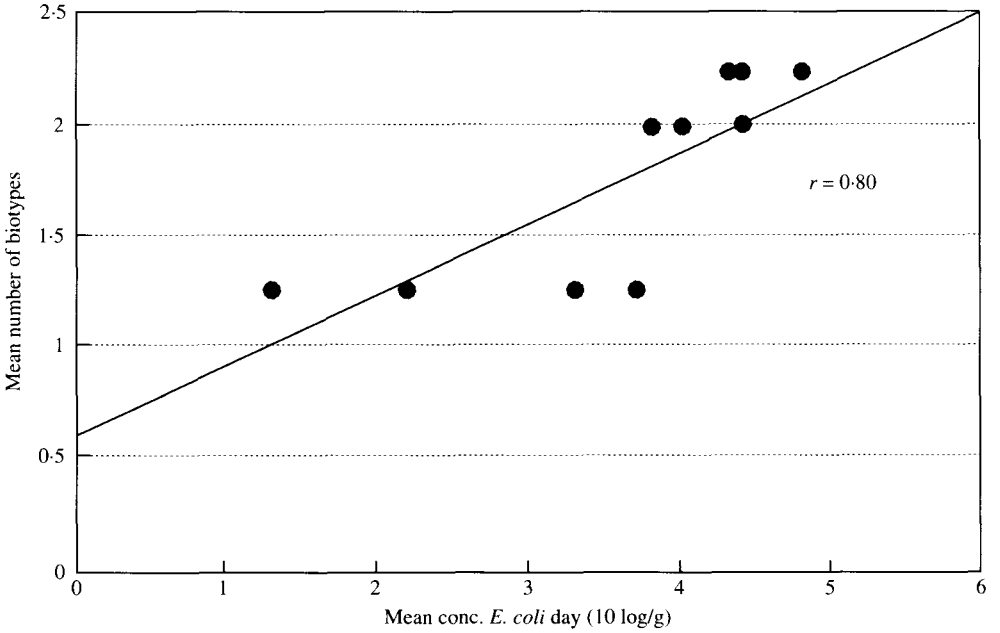


Fig. 2. Determination of the CR in 10 healthy volunteers by oral contamination with a NR-*E. coli* strain and by biotyping of faecal Enterobacteriaceae. The mean number of different biotypes is plotted on the vertical axis, the mean concentration of the NR-*E. coli* strain during the first 14 days after contamination on the horizontal axis. The two methods for determination of the CR appear to be significantly correlated ($r = 0{\cdot}80$, $P < 0{\cdot}05$).

be assessed for IgM antibody titres only (Spearman rank correlation, $P < 0{\cdot}05$), not for IgA and IgG antibody titres.

## DISCUSSION

In 10 healthy volunteers CR was measured in two ways. The first, the 'gold standard' worked out in mice, is the faecal concentration of an oral contaminating strain measured after 14 days, and the second was a measure applicable in hospitalized patients for studies of the effect of antibiotic treatment on the protective indigenous microflora [7]. Since the concentration of the oral contaminant in faeces was not constant but fluctuated, the mean concentration on days 1–14 was used instead of those on day 14. Both measures for CR were significantly correlated ($P < 0{\cdot}01$). Therefore, we decided to use biotyping of Enterobacteriaceae isolated from four faecal samples obtained within 1 week as a measure for CR instead of the ethically less acceptable method of oral contamination.

In this study, we found no correlation between concentrations of endogenous *E. coli* strains and the concentration of the NR-*E. coli* strain on day 14 after contamination. One might hypothesize that the higher the CR, the sooner a newly ingested bacterium from the environment will be eliminated from the gastrointestinal tract. However, the speed of clearance depends not only on the strain but also on the number of bacteria ingested. Ingestion of $10^9$ bacteria of a strain

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Table 2. *Serum antibody titres ($log^2$) against isolated endogenous strains of Enterobacteriaceae as well as the NR-*E. coli *strain (API 20* biotype *11144512)*

| Day | Biotypes | IgA | IgG | IgM | Biotypes | IgA | IgG | IgM |
|---|---|---|---|---|---|---|---|---|
| Volunteer A | | | | | Volunteer F | | | |
| 7 | NR-*E. coli* | < 3 | 3·1 | 3·2 | NR-*E. coli* | < 3 | < 3 | < 3 |
| 4 | NR-*E. coli* | 3·9 | 6·2 | 3·7 | NR-*E. coli* | < 3 | 4·2 | < 3 |
| 14 | NR-*E. coli* | 3·2 | 4·7 | 3·7 | NR-*E. coli* | < 3 | 3·7 | 3·8 |
| 128 | NR-*E. coli* | 4·5 | 7·7 | 5·7 | NR-*E. coli* | < 3 | 3·4 | 4·4 |
| 7 | 5044552 | 4·8 | 4·7 | 5·3 | 5144572 | < 3 | 6·3 | 6·1 |
| 7 | 7144552 | 3·8 | 6·1 | 6·0 | | | | |
| Volunteer B | | | | | Volunteer G | | | |
| 7 | NR-*E. coli* | < 3 | 4·1 | 3·4 | NR-*E. coli* | < 3 | < 3 | < 3 |
| 4 | NR-*E. coli* | < 3 | 5·1 | 3·8 | NR-*E. coli* | 3·1 | 4·8 | 3·6 |
| 14 | NR-*E. coli* | < 3 | 4·8 | 3·1 | NR-*E. coli* | 4·4 | 5·9 | 4·7 |
| 128 | NR-*E. coli* | < 3 | 6·3 | < 3 | NR-*E. coli* | 5·6 | 5·0 | 7·2 |
| 7 | 5044562 | < 3 | 3·5 | < 3 | 5044552 | 4·8 | 5·1 | 5·2 |
| 7 | | | | | 5144512 | 3·6 | 6·2 | 5·2 |
| 7 | | | | | 1144552 | 4·1 | 4·5 | 5·9 |
| Volunteer C | | | | | Volunteer H | | | |
| 7 | NR-*E. coli* | < 3 | 3·1 | 3·8 | NR-*E. coli* | < 3 | < 3 | < 3 |
| 4 | NR-*E. coli* | < 3 | 3·4 | 3·2 | NR-*E. coli* | 4·1 | 3·0 | < 3 |
| 14 | NR-*E. coli* | < 3 | < 3 | < 3 | NR-*E. coli* | < 3 | 4·9 | < 3 |
| 128 | NR-*E. coli* | < 3 | 5·4 | 3·8 | NR-*E. coli* | < 3 | 4·8 | 4·0 |
| 7 | 5144572 | < 3 | 3·2 | < 3 | 5144552 | < 3 | < 3 | < 3 |
| 7 | | | | | 5044552 | < 3 | < 3 | < 3 |
| Volunteer D | | | | | Volunteer I | | | |
| 7 | NR-*E. coli* | 5·4 | 7·7 | 6·3 | NR-*E. coli* | < 3 | < 3 | < 3 |
| 4 | NR-*E. coli* | 3·2 | 8·1 | 4·9 | NR-*E. coli* | < 3 | < 3 | < 3 |
| 14 | NR-*E. coli* | < 3 | 5·7 | 3·5 | NR-*E. coli* | 4·2 | 3·7 | 3·6 |
| 128 | NR-*E. coli* | 4·3 | 7·6 | 4·2 | NR-*E. coli* | 5·2 | 7·5 | 7·5 |
| 7 | 5004552 | 3·6 | 7·8 | 4·7 | 1044472 | 5·7 | 7·1 | < 3 |
| 7 | 5144552 | 5·6 | 8·0 | 5·7 | 5144572 | 3·6 | 6·4 | < 3 |
| 7 | 1144552 | < 3 | 5·9 | 3·8 | 1144572 | < 3 | 6·6 | < 3 |
| Volunteer E | | | | | Volunteer K | | | |
| 7 | NR-*E. coli* | < 3 | 4·2 | 4·2 | NR-*E. coli* | < 3 | < 3 | < 3 |
| 4 | NR-*E. coli* | 3·3 | 4·2 | 4·0 | NR-*E. coli* | < 3 | 3·2 | < 3 |
| 14 | NR-*E. coli* | < 3 | 3·4 | 4·4 | NR-*E. coli* | < 3 | 4·1 | 3·9 |
| 128 | NR-*E. coli* | 3·4 | 4·0 | 3·5 | NR-*E. coli* | < 3 | 4·2 | 4·8 |
| 7 | 5144552 | < 3 | 4·3 | 3·2 | 5044552 | < 3 | < 3 | < 3 |
| 7 | 5004552 | < 3 | 5·0 | 4·1 | | | | |

by the volunteers in our study may rarely occur in normal life. Indeed, the elimination of the NR-*E. coli* strain lasted quite long. Clearance of laboratory strains [10] of *Pseudomonas aeruginosa* [11] occurs within a few weeks to a few months. Van der Waaij found in five healthy volunteers, who had been given 0·5 ml of three different overnight cultures of *E. coli*, *Proteus vulgaris*, and *Enterococcus faecalis*, clearance times ranging between 5 and 14 days for the *E. coli* strain, between 0 and 7 days for the *Proteus* strain, and between 4 and 11 days for the enterococcus strain (Van der Waaij, personal communication).

There was no correlation between serum antibody titres against the NR-*E. coli*



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strain and its clearance rate from the intestines in the respective volunteers. The production of serum antibody titres against an ingested strain probably depends upon firstly, the need for the strain to colonize for a minimal period of time, and secondly, the concentration of the strain in the intestines necessary for translocation. If these two criteria are met, serum antibody titres are to be expected. From our data it can be concluded that, considering the time intervals between the clearance of the strain and the production of serum antibodies, that, regardless of their isotypes, serum antibodies are not likely to play a role in elimination of the target organism from the digestive tract. However, our results confirm that the production of circulating antibodies in higher titres may be a consequence of a (relatively) low CR.

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