J. Hyg., Camb. (1986), 96, 161–169
Printed in Great Britain

Inhibition of colonization of the chicken caecum with *Salmonella typhimurium* by pre-treatment with strains of *Escherichia coli*

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(*Received 10 June 1985; accepted 4 November 1985*)

SUMMARY

Simultaneous oral administration of broth cultures of three strains of *Escherichia coli* isolated from sewage and an abattoir strongly inhibited the colonization of a subsequently administered strain of *Salmonella typhimurium*. The three strains were protective against the *S. typhimurium* strain under a variety of conditions: in different breeds and in chickens fed different diets. The strains were not equally effective against other salmonella strains. Oral administration of the strains produced a statistically significant reduction in the excretion of the *S. typhimurium* strain over a period of 7 weeks.

INTRODUCTION

The much greater susceptibility of the newly hatched chicken to oral infection by food-poisoning salmonellas when compared with adult chickens is attributed to the virtual absence of an intestinal microflora (Millner & Shaffer, 1952; Nurmi & Rantala, 1973). The resistance of the adult can be conferred on newly hatched chickens by oral administration of a suspension of faeces or caecal contents obtained from adult chickens (Nurmi & Rantala, 1973; Rantala & Nurmi, 1973). This 'competitive exclusion' has been confirmed by many groups of workers (Lloyd, Cumming & Kent, 1977; Snoeyenbos, Weinack & Smyser, 1978; Barnes, Impey & Cooper, 1980; Dorn & Krabisch, 1981) as a potential means of reducing the incidence of intestinal infections with food-poisoning salmonellas in commercial poultry.

Although the inhibitory effect on salmonellas can be reproduced by anaerobic cultures of faeces or caecal contents (Rantala, 1974; Snoeyenbos, Weinack & Smyser, 1978) one of the problems inherent in the widespread commercial application of such preparations is the risk of simultaneous inoculation with other avian pathogens present in the alimentary tract of the poultry from which the intestinal contents were obtained. One of the ways in which this problem can be overcome is to administer a mixture of pure cultures of micro-organisms obtained from the same source. Barnes, Impey & Cooper (1980) and Impey, Mead & George (1982) were able to induce protection against salmonella infection in young chicks using a pool of cultures of both obligate and facultative anaerobes obtained from chickens. Although these workers have found that simultaneous administration of a relatively large number of bacterial strains was necessary to produce

protection, others have found that individual strains were effective. Soerjadi, Lloyd & Cumming (1978) used a strain of *Streptococcus faecalis* and Rigby, Pettit & Robertson (1977) used an unidentified strain of *Clostridium*, both groups reporting a protective effect from their cultures.

So far there are no reports of protection induced by strains of facultatively anaerobic Gram-negative bacteria. Previous reports (Idziak & Caldwell, 1977; Snoeyenbos, Weinack & Smyser, 1978) have indicated that strains of *Escherichia coli* were ineffective. However, the sources of these strains were not stated and it was considered that protective organisms of this sort might be found in environments in which coliform organisms co-exist with enteric pathogens. The aim of the present work was to find a 'salmonella-like' organism which might occupy the same niche as salmonellas and thus inhibit their colonization without possessing their virulence attributes.

An organism which induced protection when administered on its own was not found. However, three strains of *E. coli* were isolated from sewage and an abattoir drain which when inoculated orally into newly hatched chickens were inhibitory for salmonella organisms. This paper describes their isolation, the extent of their protective effect and an assessment of their practical significance.

MATERIALS AND METHODS

Chickens

Unless otherwise stated these were from a specified-pathogen-free Light Sussex flock. Their sex was not determined. Their rearing conditions and diet have been described previously (Smith & Tucker, 1975).

Bacteria

Broth cultures of all strains were made in 10 ml nutrient broth (Oxoid CM67), incubated for 24 h at 37 °C in a shaking water bath. They contained between 1 and 3×10^9 c.f.u./ml. In most experiments a spontaneous mutant resistant to nalidixic acid (Nal^r) of a strain of *Salmonella typhimurium*, F98 of phage type 14 was used as the challenge organism.

Bacteria isolated from environmental and faecal samples and tested for salmonella-inhibitory activity were identified using the API 20E identification system (API products, France). They were screened for selected virulence determinants as follows. Haemolysin production was assessed by growing strains on plates of nutrient agar containing 7 % washed sheep erythrocytes. Heat-labile enterotoxin (LT) was detected in rabbit ligated intestine (Smith & Linggood, 1971) and heat-stable enterotoxin (ST) in pig ligated intestine (Smith & Gyles, 1970). Vero cell toxin was detected by the method of Konowalchuk, Speirs & Stavric, (1977) and colicin V production by the method of Smith & Huggins (1976).

Procedure for isolating bacteria from environmental samples that inhibited colonization of the chicken caeca by S. typhimurium *F98 Nal^r*

Environmental and faecal samples were tested as aqueous suspensions for the ability to inhibit *S. typhimurium* F98 Nal^r colonization by the following method: 0·1 ml of the sample was inoculated orally into the crop of a group of 10 chickens within 24 h of hatching and immediately before the chickens were provided with food. This was followed 24 h later by 0.1 ml of a broth culture of F98 Nal^r, diluted so as to contain 10^5 viable organisms. The chickens were killed 3 days later and swabs of their caecal contents were cultured on plates of Brilliant Green agar (Oxoid CM263) containing sodium nalidixate (20 μg/ml) and novobiocin (1 μg/ml). This procedure was performed in a standard manner so that an impression could be gained of the relative numbers of the salmonella organisms in the caecal contents of the chickens (Smith & Tucker, 1975). The results were then compared with those for a group of chickens that had been given F98 Nal^r only.

The caecal swabs were stored at 4 °C overnight and those from chickens given samples that inhibited colonization with F98 Nal^r were then cultured on several types of media including MacConkey agar (Oxoid CM7), Brilliant Green agar, Tryptose agar to show swarming organisms (Difco) and blood agar to show haemolytic organisms (Blood agar Base (Oxoid CM55) containing 7% citrated sheep blood). These were incubated aerobically and up to 50 colonies, including all those of differing morphology, were picked and purified twice on MacConkey agar. These were tested by slide agglutination with salmonella poly-O antiserum (Wellcome) to ensure that none was a salmonella. The organisms were grown in nutrient broth and the resulting cultures pooled in equal amounts and the pool tested in chickens for anti-salmonella colonizing activity in the usual manner. If the pool was found to possess such activity smaller groups of cultures were tested with a view of determining which strain or strains were responsible for the inhibition.

Attempts were then made to improve any mixture of organisms which was found to be inhibitory. This was done by inoculating a mixture of cultures of these strains together with a pool of organisms obtained from another inhibitory sample and repeating the procedure.

Procedure for assessing the inhibitory activity of bacteria isolated from environmental and faecal samples

Broth cultures of inhibitory organisms were mixed in equal amounts and inoculated into young chicks as described above. They were challenged and killed in the same way. Viable counts of caecal contents were performed by a modification of the method of Miles & Misra (1938) using Brilliant Green agar containing sodium nalidixate and novobiocin.

For the long-term assessment of inhibition of salmonella excretion the method of Smith & Tucker (1975) employing cultivation of caecal swabs on agar with selective enrichment in sodium selenite broth (Oxoid CM395) was used.

RESULTS

The inhibition of caecal colonization by S. typhimurium *F98 Nal^r by bacteria from environmental and faecal samples.*

In all, 109 samples were examined, singly or pooled, for the ability to inhibit caecal colonization by *S. typhimurium* F98. These included faecal samples from cattle, sheep, pigs, chickens and wild birds, samples from sewage works, abattoir and animal market drains, and manure and compost heaps, river and stagnant water, decaying vegetation and soil. Of these, 22 of the 27 sewage samples, 14 of the 25 faeces samples from avian species, 0 of the 9 faeces samples from other animals and 1 of the 48 environmental samples inhibited salmonella colonization. Three strains of *E. coli* were isolated from sewage samples which, when inoculated as a pool, were inhibitory for *S. typhimurium* F98 Nal^r. Attempts were made to increase the inhibitory effect of the three strains by adding to the pool bacterial strains from other inhibitory samples. One strain, isolated from an abattoir drain was found to augment their effect. In doing so, one of the original three strains became superfluous.

During their isolation the finally selected three strains were designated 5, 10 and 14. All three were identified as *E. coli*. Dr B. Rowe of the Division of Enteric Pathogens, Central Public Health Laboratory, Colindale revealed that none of them possessed a recognizable O antigen. None of them produced any of the virulence determinants for which they were examined, i.e. haemolysin, LT, ST, Vero toxin or colicin V. Oral inoculation of a pool of broth cultures of the three strains into 30 day-old chickens produced no disease or mortality. Oral inoculation of a human being with 10^9 organisms of each strain in 10 ml broth produced no ill effects.

The effect of pre-treatment with E. coli *strains 5, 10 and 14 on the numbers of* S. typhimurium *F98 Nal^r organisms in the caecal contents of chickens*

The effectiveness of the three strains of *E. coli* in reducing the viable counts of *S. typhimurium* F98 Nal^r in the caecal contents of chickens was assessed on 13 different occasions and the results were pooled (Table 1). In the control group, not pre-treated with *E. coli* strains 5, 10 and 14, most of the caecal counts of F98 Nal^r were very high. Of the counts 87 % were greater than 10^6. Compared with this group there was considerable inhibition of F98 Nal^r in the chickens pre-treated with the three strains. While 15 % of the counts were still greater than 10^6/g, 30 % of the counts were less than 10^2/g compared with less than 2 % of the untreated chickens.

The three strains produced similar inhibitory effects against F98 Nal^r when tested in chickens reared on five different diets, including three commercially available types and when tested in four other breeds of chickens, these being Rhode Island Red, White Leghorn, Brown Leghorn and a commercial broiler breed.

The effect of E. coli *strains 5, 10 and 14 on different salmonella serotypes and phage types of* S. typhimurium

The results of challenging groups of pre-treated and untreated chickens with 10 strains of *S. typhimurium* of 9 phage types and 5 strains of other serotypes are presented in Table 2. Appreciable reductions in the caecal salmonella counts were

Table 1. *The effect of pre-treatment with* E. coli *strains 5, 10 and 14 on caecal colonization by* S. typhimurium *F98 Nal*^{r}

Viable counts of F98 Nal^r/g of caecal contents	The numbers of chickens with specified viable counts of *S. typhimurium* F98 Nal^r in the caecal contents after or without pre-treatment with	
	E. coli strains 5, 10 and 14	Nothing
$> 10^8$	1	33
10^6–10^8	21	88
10^4–10^6	41	12
10^2–10^4	31	4
$< 10^2$	46	2
Total	140	139

Table 2. *The numbers of salmonella organisms in the caecal contents of groups of 10 chickens given different salmonella strains one day after* Escherichia coli *strains 5, 10 and 14*

	Log_{10} no. viable salmonella organisms/g of caecal contents of chickens that were	
	Pre-treated with *E. coli* strains 5, 10 and 14	Untreated
Salmonella typhimurium		
Phage type 44	3·6 (< 2·0–8·5)	7·6 (2·5–7·9)
Phage type 14 (F98)	4·3 (< 2·0–7·4)	7·7 (3·6–8·6)
Phage type 40	5·1 (3·5–6·8)	7·4 (6·3–8·2)
Phage type 10	6·0 (3·3–8·4)	7·4 (3·7–7·7)
Phage type 36	6·9 (2·7–7·6)	7·6 (5·3–8·3)
Phage type 49	7·3 (< 2·0–8·6)	8·3 (7·9–8·6)
Phage type 1	7·3 (3·6–8·8)	7·3 (6·6–7·6)
Phage type 20 (strain A)	8·1 (6·1–8·4)	8·6 (7·6–9·0)
Phage type 20 (strain B)	7·7 (6·9–8·3)	7·9 (6·2–8·5)
Phage type 104	7·2 (6·0–8·4)	6·4 (< 2·0–8·2)
S. agona	4·9 (3·0–8·3)	8·3 (5·9–9·1)
S. enteritidis	7·4 (3·2–8·9)	7·9 (7·5–8·3)
S. anatum	7·7 (3·2–8·9)	8·4 (7·4–9·1)
S. hadar	7·9 (3·2–7·3)	8·3 (7·6–8·5)
S. infantis	8·1 (3·4–8·8)	8·4 (3·4–8·7)

The median count is given followed by the range in parentheses.
10 chickens in each group.

obtained in chickens given 6 of the 10 strains of *S. typhimurium* and the *S. agona* strain. In the chickens given the other 8 strains little or no reduction was observed.

The faecal excretion of S. typhimurium *F98 Nal*^{r} *by chickens previously inoculated with strains 5, 10 and 14*

The results of examining the faeces of groups of 50 chickens at frequent intervals after they had been given strains 5, 10 and 14 or no treatment and then infected 24 h later with 10^5 organisms of *S. typhimurium* F98 Nal^r are shown in Table 3.

https://doi.org/10.1017/S0022172400065931 Published online by Cambridge University Press

Table 3. *The isolation of* S. typhimurium *F98 Nal^r from the faeces of two groups of 50 chickens pre-treated with* E. coli *strains 5, 10 and 14 or left untreated and both inoculated 24 h later with 10^5 organisms of F98 Nal^r*

	% of 50 chickens from which F98 Nal^r was isolated after inoculation with					
	E. coli strains 5, 10 and 14			Nothing		
Days after pre-treatment	> 50*	D	T	> 50*	D	T
7	4	12	22	6	28	48
14	2	8	20	10	34	58
21	2	14	26	18	52	74
28	0	10	26	6	26	54
35	0	8	14	2	22	42
42	0	4	6	2	6	20
49		2	2	0	6	20
49 (caeca) †	0	0	0	0	6	14

* > 50, 50 or more salmonella colonies after direct plating; D, 1 or more salmonella colonies after direct plating; T, salmonella organisms isolated by direct plating or after selenite enrichment.

† Slaughter at 49 days.

Each group of chickens was housed in a separate room.

There was a considerable reduction in the excretion rate of the salmonella strain in those chickens pre-treated with strains 5, 10 and 14 from soon after challenge. There was a reduction both in the number of chickens from which salmonellas were isolated and in the number of chickens from which large numbers of salmonellas were isolated. At slaughter, 48 days after challenge with the salmonella, 14% of the untreated chickens harboured F98 Nal^r in their caeca whereas the pre-treated chickens were free of infection. The differences in the rate of isolation of F98 between the two groups during the course of the experiment was statistically significant ($\chi^2 = 15{\cdot}3$, $P = 0{\cdot}03$).

DISCUSSION

Although, by using the methods we described, we were unable to isolate a strain of Gram-negative facultative anaerobe which was inhibitory for salmonellas when inoculated on its own we found that a mixture of three strains of bacteria, identified as *E. coli*, produced a profound reduction in the caecal count of *S. typhimurium* F98, a strain used by ourselves in previous studies (Smith & Tucker, 1975; Smith & Tucker, 1980) and also by other workers (Impey, Mead & George, 1982). Good protection against colonization of the caeca by this strain was also obtained in chickens reared on different diets and in different breeds of chickens, including a commercial broiler breed.

The three strains of *E. coli* were isolated from sewage and from an environmental sample (abattoir drain). While avian faecal samples were protective *per se* we were unable to isolate inhibitory facultative anaerobes from them. It was likely that in these samples protection against salmonella colonization was mediated primarily by obligate anaerobes as in the original competitive exclusion system. That this

is so is suggested by the fact that faeces from other animals were completely ineffective, such specificity having already been observed (Rantala & Nurmi, 1973). This result does suggest that chicken intestinal contents are not necessarily the best samples from which to isolate inhibitory coliforms.

Inhibition of the salmonella strain was not consistent from chicken to chicken, some of the caecal salmonella counts of pre-treated birds being still quite high. This absence of complete protection has also been observed using the system involving pre-treatment with faeces or caecal contents obtained from adult chickens (Nurmi & Rantala, 1973; Rantala, 1974; Impey, Mead & George, 1982). It was considered that, since strains of *E. coli* were being used for pre-treatment, interference of the colonization of these organisms by *E. coli* strains already in the alimentary tract may have occurred leading to poor establishment of the protective strains. This is a distinct possibility since *E. coli* is known to be one of the first micro-organisms to colonize the gut of the chicken after hatching (Smith, 1965; Barnes, Impey & Cooper, 1980; Coloe, Bagust & Ireland, 1984). For our experiments chickens were inoculated with the mixture of protective strains approximately 24 h after hatching began. Some of the chickens would have been almost 1-day-old at the time of pre-treatment and could have had considerable numbers of *E. coli* in their alimentary tracts. This problem could be easily overcome, however, by ensuring that the protective organisms are ingested before any others and could be accomplished, for example, by spraying the eggs with bacterial cultures shortly before hatching commences.

The mechanism of inhibition was not elucidated. Other workers had suggested that the protective effect of the complex mixture of obligate and facultative anaerobes of caecal contents and faeces is mediated by blocking adhesion sites on the wall of the alimentary tract which are essential for salmonella colonization (Snoeyenbos, Weinack & Smyser, 1979). The fact that no single *E. coli* strain was found which influenced the colonization of the caeca by the *S. typhimurium* strain, while a mixture of three *E. coli* strains usually had a profound effect makes it unlikely that the three strains did so by occupying salmonella attachment sites. It is more likely that they achieved their effect by each 'blocking' a different part of an essential salmonella metabolic pathway. That they were not equally effective against other salmonella strains tested either suggested that different colonization mechanisms might be involved or that strains differed in their relative ability to colonize the alimentary tract. Some of these strains had been deliberately chosen for inclusion in these studies because Smith & Tucker (1980) had previously shown them to be better colonies than *S. typhimurium* F98.

The reduction in faecal excretion of this salmonella strain persisted until 50 days, an age when broilers are frequently killed indicating that such a system could be useful under commercial conditions. At slaughter the caeca of the pre-treated chickens were completely free of infection whereas $\frac{1}{7}$ of untreated chickens still harboured salmonella in their caeca.

It is suggested that there are practical advantages in using a small pool of Gram-negative facultatively anaerobic bacteria to inhibit salmonella in poultry and that a wider search might produce strains which were protective against the salmonella types which were refractory to the inhibitory effects of the *E. coli* strains described in this study. In the long term, however, it would seem more logical to

attempt a better understanding of the mechanisms by which food-poisoning salmonellas colonize the alimentary tract of the chicken in order that searches can be made for organisms with defined characteristics rather than discovering them empirically.

We are grateful to Mrs J. Simpson and Mrs K. Megson for capable technical help. Our thanks are also due to Professor P. M. Biggs, Dr H. Williams Smith, Dr G. C. Mead, Mrs S. Lewin and Mrs S. Pilcher for assistance in various ways.

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