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Role of Adhering Microflora in Competitive Exclusion of *Salmonella* from Young Chicks

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ABSTRACT

Adherence of gut microflora from *Salmonella*-free adult birds to the ceca of newly hatched chicks, and their role in protection against *Salmonella* was examined. The protective microflora remained attached to the cecal wall after four successive washings. Cultures of washed ceca taken from chicks 30 min to 1 h after treatment with fecal culture gave partial protection. Full protection was obtained with cultures from ceca taken 6 to 8 h after treatment.

Protection of chicks against infection with *Salmonella* by the native gut microflora from *Salmonella*-free adult birds has been firmly established (cited in *16)*. Several mechanisms have been postulated by which native gut microorganisms prevent infection *(11,12)*. Of particular interest is the proposed competitive exclusion mechanism *(7)* by which *Salmonella* and native gut flora compete for sites of adherence on the intestinal wall of young chicks. Adherence as a prerequisite to invasion by enteropathogens requires access to receptors on host tissue. Thus firm evidence for epithelial adherence by native bacteria would lend support to the concept that receptor occupation could be part of their protective role *(4)*.

Adhering bacteria have been defined as those that are not readily removed from the epithelium by specified washing procedures *(9)*. The role of adhering bacteria in protection against enteric pathogens was recognized by Fuller *(3)*, who suggested treating newly hatched chicks with chicken feces or pure cultures of adhering lactobacilli. The same year Nurmi and Rantala *(8)* prevented infection with *Salmonella* in young chicks by treatment with cultured chicken feces. Using a specified washing procedure and electron microscopy, Soerjadi et al. *(14)* found that native gut flora from donor birds adhered to the epithelial surface of the ceca in young chicks, and that the crop and ceca were the major sites for *Salmonella* colonization. Similarly, in germ-free chicks treated with *Salmonella* the highest *Salmonella* count was observed in the ceca *(6)*.

Resistance of chicks to *Salmonella* infection increased with time after treatment with fecal material. Some protection was obtained within 1 to 2 h *(13)*, but maximum protection was not obtained until 24 to 48 h after treatment *(14,15)*.

We have used the cecal content of *Salmonella*-free donor birds as a source for isolation of protective bacteria *(16)*. In this work we examined the possibility of using washed ceca of young chicks, treated with fecal culture, as a source for anti-*Salmonella* treatment. We assumed that native gut flora, which plays a role in protection, firmly adhered to the cecal wall of treated chicks. Washing of the ceca would eliminate non-adherent bacteria and thus serve as a screening process. Such ceca could then be used as a selective source for the isolation of protective bacteria. We also examined the time period that was necessary for adhesion of the protective bacteria.

MATERIALS AND METHODS

General experimental conditions

Preparation of fecal culture and mixtures of pure cultures. Fecal cultures (FC) for treatment were prepared by inoculating 0.1 g of fresh feces from *Salmonella*-free donor birds into 100 ml of VL medium *(1)* and incubating for 3 d at 37°C. Mixtures of pure cultures (PC) *(16)* were prepared from frozen or lyophilized stock cultures which were streaked on Columbia blood agar plates and incubated for 3 d at 37°C. Several colonies of each culture were then used to inoculate a common bottle of VL medium which was incubated for another day at 37°C. All cultures were incubated anaerobically.

Standard laboratory trials. Chicks (White Rock × Cornish) used in the trials were always supplied by Curtiss Chicks Ltd., Port Hope, Ontario. Chicks (Leghorn) used for preparation of washed and whole ceca were sometimes hatched in our facilities.

One-day-old chicks (day of hatch) were treated per os with 0.5 ml of treatment culture and challenged 2 d later in the drinking water with 10^5 colony-forming units of nalidixic acid-resistant (Nal^+) *Salmonella typhimurium* per chick *(2)*. Ten chicks per treatment, usually in duplicate, were housed in wire-floored starter brooders where feed and water were always available. Six days after challenge, cecal contents were examined for *Salmonella* infection by the swab-plate method *(2)*.

Protection was assessed by the criteria proposed by Pivnick et al. *(10)*. These were: *(a)* number of chicks with different

levels of *Salmonella* per g of cecal content *(2)*, *(b)* percentage of birds with infection, *(c)* Infection Factor (IF), and *(d)* Protection Factor (PF). Infection Factor takes into account the degree of infection and is the mean of the $\log_{10}$ values of the numbers of *Salmonella* per g of cecal content of all chicks treated and challenged identically. PF is the ratio of IF for the untreated group of chicks over IF for the treated group and thus expresses the efficacy of treatment. A PF value exceeding 25.0 (>25.0) signifies essentially full protection while a value of <4.0 shows low or no protection.

Preparation of ceca as inocula for treatment against Salmonella

Passaging. On the day of hatch, chicks were orally treated with fecal culture (FC chicks) or mixtures of pure cultures (PC chicks). Chicks (usually 8 in each treated and non-treated control group) were reared in wire starter brooders and provided with feed and water. They were sacrificed by cervical dislocation, usually 3 or 14 d after treatment, unless stated otherwise.

Washing of ceca. One of the two ceca from each treated chick and from each non-treated chick was washed, while the other was used intact (whole ceca). These preparations were done aseptically under anaerobic conditions in a double-port anaerobic chamber (Coy Lab. Prod. Inc., Ann Arbor, Mich.). Washed ceca were prepared by a modification of the method of Soerjadi et al. *(14)*. The distal end of each cecum was inverted on a wooden stick and placed in a dilution bottle (4 ceca/bottle) containing 100 ml of reduced phosphate buffered saline solution (PBS). The bottles were gently shaken (inverted) 10 times and left standing for 2 min before the ceca were transferred to another bottle. There were four successive washings by the same procedure. Washed or whole ceca were either homogenized after washing and used immediately, or were stored in 15% glycerol/anaerobic dilution solution *(8)* at -60°C and homogenized when required. If used immediately, the washed or whole ceca were homogenized in VL medium in a Sorval Omni Mixer for 2 min at high speed (16,000 rpm). Portions of the homogenates were inoculated into VL medium which was incubated at 37°C for 72 h before use as a treatment for 1-d-old chicks.

RESULTS AND DISCUSSION

Adherence of protective microflora

Effect of washing on protective activity of ceca. Table 1 compares the protective activity of cultures of washed and whole ceca, and of feces, all from adult donor birds. At a low dilution of inoculum (10^{-4} g or lower) cultures of washed ceca gave essentially full protection comparable to cultures of feces and whole ceca, while at the higher dilutions washed ceca were less protective. This might be expected because the inoculum was prepared on a weight basis. Whole ceca and feces would contain a larger assortment and number of bacteria than washed ceca. As the quantity of inoculum was decreased there was a gradual loss of protective activity from all three cultured preparations indicating that not all species of bacteria needed for starting fully protective cultures were present at higher dilutions. The results with washed ceca at low dilutions of inoculum showed that bacteria relevant to protection resisted the washing procedure and presumably remained adherent to the cecal wall.

Use of washed and whole ceca as a treatment. In the following experiments the ceca were obtained from young chicks that had received fecal culture or mixtures of pure cultures (FC and PC chicks). Cultures of washed ceca taken from FC chicks 3 and 14 d after treatment offered essentially full protection (PF >25.0) (Table 2), whereas cultures of washed ceca from control chicks did not protect chicks (PF <2). Considerable protection was obtained with cultures of whole ceca from 14-d-old control chicks (PF 10.1), but no protection (PF 1.7) from 3-d-old chicks. It is likely that control chicks developed some protective flora by 14 d of age, but this flora was less protective than the flora from FC chicks. Since comparable cultures of washed ceca had no protective effect, it is possible that the acquired microflora either did not adhere to the cecal wall, or that adherence was weak.

TABLE 1. *Comparison of protective activity of cultures*[a] *from washed ceca, whole ceca and feces of adult donor birds against* Salmonella.

Grams of inoculum per treatment culture	Washed ceca			Whole ceca			Feces		
	Percent infection	Infection[b] factor	Protection[c] factor	Percent infection	Infection factor	Protection factor	Percent infection	Infection factor	Protection factor
10^{-3}	5	0.05	>25.0	0	<0.1	>25.0	10	0.25	>25.0
10^{-4}	5	0.05	>25.0	0	<0.1	>25.0	5	0.05	>25.0
10^{-5}	37	0.9	4.9	5	0.05	>25.0	10	0.1	>25.0
10^{-6}	25	0.8	5.5	5	0.05	>25.0	5	0.2	>25.0
10^{-7}	30	1.4	3.1	5	0.05	>25.0	20	0.8	8.3
10^{-8}	57	1.9	2.3	35	1.6	3.5	35	2.0	3.3
non-treated (control)	95	4.4		100	5.6		100	6.6	

[a]Each 3-d-old culture was tested for protective activity by introducing 0.5 ml of a 1:10 dilution into the crop of 10 1-d-old chicks (in duplicate) and assessed by standard laboratory trials (see Materials and Methods). Results are averages of two trials, each trial with 20 chicks.

[b]"Infection factor" (the mean $\log_{10}$ value of the numbers of *Salmonella*/g of cecal content for a group of chicks treated and challenged identically).

[c]"Protection factor" (ratio of IF for untreated/treated chicks).

TABLE 2. *Protection of chicks against* Salmonella *with cultures of washed and whole ceca from chicks in which fecal culture was passaged.*

Passaging of fecal culture in chicks whose ceca were used as inocula for treatment cultures		Efficacy of treatment cultures			
Fecal culture introduced at 0 d	Age of chicks when ceca were excised (d)	No. of chicks	Percent infection	Infection[d] factor	Protection[e] factor
Washed ceca					
Yes[a]	3	80	3	<0.1	>25.0
	14	80	8	0.1	>25.0
No[b]	3	79	71	3.3	1.4
	14	80	74	2.9	1.6
Whole ceca					
Yes[a]	3	40	0	<0.1	>25.0
	14	40	3	<0.1	>25.0
No[b]	3	40	73	2.8	1.7
	14	40	15	0.4	10.1
Experimental controls[c]					
Fecal culture		60	5	<0.1	>25.0
Control		60	97	4.6	

[a]Newly hatched chicks were given fecal culture per os. After 3 and 14 d chicks were sacrificed, ceca washed or left intact, homogenized and cultured for 72 h. These cultures were used as the treatment against *Salmonella* in 1-d-old chicks. Results are averages of 2 to 4 trials.

[b]The same as in [a], but chicks were not given fecal culture.

[c]Fresh fecal culture was used for treatment in each trial; control chicks were not treated.

[d,e]See footnotes b,c of Table 1.

When PC chicks were used instead of FC chicks, the cultures of both washed and whole ceca taken 3 or 14 d after treatment, offered essentially the same protection (Table 3). This again confirms adherence of PC to the cecal wall. However, after a 3-d passage, less protection was obtained with cultures of both washed and whole ceca than with the same cultures from 3-d-old FC chicks (Table 2). This suggests that both defined mixtures were still lacking some important organism(s) or factor(s). The large difference in protection obtained with cultures of washed and whole ceca between 3-d (av. PF 7.1) and 14-3-old PC chicks (av. PF>25.0) indicated that some of these missing componens may have been acquired naturally by 14 d of age. We have no explanation for essentially the same results obtained with 44 and 28 PC mixtures.

Time study

Effect of time on development of protective microflora. Table 4 shows that cultures from washed ceca of chicks sacrificed 30 min or 1 h after administration of fecal culture had already some protective activity (PF 6.1), and that the protective flora started to adhere to the cecal wall almost immediately. After 6 to 8 h all species of bacteria necessary for starting protective culture appeared to be attached. The time needed for their establishment was considerably shorter than that reported by Soerjedi et al. *(14)* and Seuna *(13)* but their experimental conditions differed from ours.

Effect of culturing on protective activity of washed ceca. In all the previous experiments, washed ceca were cultured for 3 d before they were used as treatments against *Salmonella* infection. In subsequent experiments we examined whether culturing had an effect on the potency of washed ceca. Table 5 shows the protective activity of non-cultured washed ceca. Some protection was evident with washed ceca from chicks in which fecal culture was passaged for 6 h, whereas full protection was obtained after a passage time of 4 d. Since cultures of washed ceca from 6-h FC chicks also provided nearly full protection (Table 4), these results show that a passage time of 6 h was adequate for essentially all protective bacteria to adhere to the cecal wall, though not in sufficient quantity, and that a longer passage time was required for the protective microflora to adhere in adequate numbers (Table 5).

We have demonstrated that the protective flora from fecal culture started to adhere almost immediately to the cecal wall of newly hatched chicks, and that all bacteria relevant to protection were represented in the adhering microflora 6 to 8 h after administration. The data also showed that washed ceca of chicks treated with fecal culture for a short period would not be a good source of protective bacteria.

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TABLE 3. *Protection of chicks against* Salmonella *with cultures of washed and whole ceca from chicks in which mixture of pure cultures was passaged.*

Passaging of mixtures of pure cultures in chicks whose ceca were used as inocula for treatment cultures			Efficacy of treatment cultures		
Mixture of pure cultures introduced at 0 d		Ages of chicks when ceca were excised (d)	Percent infection	Infection[d] factor	Protection[e] factor
Washed ceca					
28 PC[c]		3	15	0.5	7.6
44 PC	Yes[a]	3	15	0.6	7.3
28 PC		14	5	0.2	21.0
44 PC		14	10	0.1	>25.0
	No[b]	3	71	3.3	1.4
		14	74	2.9	1.6
Whole ceca					
28 PC		3	34	0.6	7.0
44 PC	Yes	3	30	0.6	6.5
28 PC		14	5	0.1	>25.0
	No	3	73	2.8	1.7
		14	15	0.4	10.1
Experimental controls					
Fecal cultures			0	<0.01	>25.0
Control			95	4.4	

[a]Newly hatched chicks were given mixtures containing 28 or 44 pure cultures. After 3 and 14 d chicks were sacrificed, ceca washed or left intact, homogenized and cultured for 72 h. These cultures were used as the treatment against *Salmonella* in 1-d-old chicks. Results are averages of two trials.

[b]The same as in [a], but chicks were not given mixtures of pure cultures.

[c]Number of pure cultures in a mixture.

[d,e]See footnotes b,c of Table 1.

TABLE 4. *The effect of duration of passage[a] of fecal cultures in chicks on the suitability of their washed ceca as inocula for anti-*Salmonella *treatment cultures.*

Duration of passage (h)	Percent infection	Infection[c] factor	Protection[d] factor
0	85	3.9	1.1
0.5	31	0.7	6.1
1	23	0.7	6.1
3	20	0.5	7.7
6	8	0.2	21.3
8	0	0	>25.0
18	0	0	>25.0
24	5	0.2	21.3
48	15	0.2	21.3
72	3	<0.1	>25.0
336	8	0.1	>25.0
Non-treated[b] (ceca washed at 48 h)	85	4.1	1.0
Control	90	4.3	

[a]Fecal culture was passaged for certain time in newly hatched chicks, then the ceca were excised, washed, homogenized and cultured for 72 h in VL medium. The cultures were assayed for anti-*Salmonella* activity in 1-d-old chicks. Results are averages from two trials.

[b]The same as in [a], but chicks were not given fecal cultures.

[c,d]See footnotes b,c of Table 1.

TABLE 5. *Protective activity of non-cultured[a] washed ceca from chicks in which fecal culture was passaged.*

Passaging of fecal culture (time)	No. of chicks	Percent infection	Infection[b] factor	Protection[c] factor
6 h	20	55	1.6	2.6
4 d	30	3	<0.1	>25.0
Control	20	95	4.2	

[a]Fecal culture was passaged for 6 h and 4 d in newly hatched chicks. Then the ceca were excised, washed, resuspended in VL medium and used as the treatment against *Salmonella* in 1-d-old chicks.

[b,c]See footnotes b,c of Table 1.

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Jermini et al., *con't. from p. 927*

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