J. Hyg., Camb. (1968), **66**, 207
Printed in Great Britain

Age susceptibility and excretion of *Salmonella typhimurium* in calves

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(*Received* 14 *October* 1967)

The production and sale of surplus dairy calves may involve transport, marketing, slaughter, or rearing on a new property. Economics dictate that these transfers take place at a young age, usually 4–6 days. The problem of cross-infection at this time, particularly with salmonella organisms, has been recognized for some time (Anderson, Galbraith & Taylor, 1961; Robinson, 1966). Surveys (Anon. 1964) have shown that the incidence of clinical salmonellosis in purchased calves is considerably higher than in home-bred calves. It requires only a few calves excreting salmonellas on the farm to result in a high isolation rate of organisms following slaughter. Factors which contribute to this rapid build-up of infection include time of transport and holding, number of animals per unit area, partial colostrum deprivation, starvation conducive to extensive sucking amongst calves, and passage of calves through contaminated environments. De Jong & Ekdahl (1965), as a result of observations on the response of calves to *S. typhimurium* have suggested that if animals were retained on the farm of birth until they were at least 14 days of age, the prevalence of salmonella infection amongst calves entering the abattoir might well be reduced. Others (Williams Smith, 1966; Walton, 1966) have made similar suggestions in support of a ban on the movement of calves less than 6 weeks old.

Robinson (1966) has observed that calves may be exposed to infection within the first few hours of birth, either from a contaminated environment or from the adult cow. Excretion of salmonellas in the milk does not commonly occur unless the cow is clinically affected with salmonellosis, but gross contamination of the udder and teat surfaces with salmonellas can be demonstrated both in asymptomatic excreting animals and in recently calved cattle held in a contaminated environment.

In view of the above suggestions, it was, therefore, considered useful to compare the response of two groups of calves to several dose rates of *S. typhimurium* given orally at approximately 2 days of age, and at 14–21 days of age. These will be referred to as 'younger' and 'older' groups respectively. In these calves, the concentration of *S. typhimurium* in the faeces, the duration of faecal excretion and the

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sites of recovery of the organisms after slaughter were examined. Calves were given doses of organisms which were unlikely to result in clinical disease. Throughout these trials calves were fed on whole raw milk. This would not be an economic feeding regime, but it was thought that data should be obtained on the response of calves under optimum nutritional conditions. The use of milk substitutes, partial colostrum deprivation and temporary starvation, are all additional factors which warrant separate investigation in the response of young calves to salmonella infections.

METHODS AND MATERIALS

Preparation of salmonella suspensions

Lyophilized cultures of the calf-passaged strain of *S. typhimurium* (phage-type 1) used by de Jong and Ekdahl were reconstituted in 0·5 ml. tryptose phosphate broth* and inoculated on 8 ml. nutrient agar slopes. These were incubated at 37° C. for 18 hr. and the bacterial growth was then washed off with 2 ml. broth and appropriate dilutions made in quarter strength Ringer solution. Viability counts were made by spreading 0·1 ml. volumes of dilutions on MacConkey agar plates in duplicate. A fresh freeze-dried culture was used for each group of animals. The required dose was given in 40 ml. sterile milk by mouth midway between morning and afternoon feeds.

Isolation of salmonellas

Apart from enumeration of the number of salmonellas in faecal samples, all materials were cultured for 24–30 hr. at 37° C. in tetrathionate broth, containing brilliant green at a final concentration of 1/100,000. Subcultures were made on freshly poured brilliant green (BG) agar with 8 mg. sulphadiazine added per 100 ml. agar. These plates were incubated for at least 24 hr. before picking suspicious colonies to a tube each of triple sugar iron agar (TSI) and lysine broth. BG plates with scanty growth were not discarded as negative until after 48 hr. incubation. All suspicious TSI tubes were confirmed serologically. The results of Nottingham's (1967) studies on six enrichment and plating media combinations showed the above method was the most efficient, obtaining 82% of the total salmonella isolations from naturally infected calf tissues.

Faecal samples

All calves were sampled daily until slaughter; faeces for quantitative estimation of salmonellas was obtained by using sterile wooden spatulas, but later qualitative examinations were made using rectal calcium alginate wool swabs only. Enumeration of the number of salmonellas/g. of faeces (wet weight) was carried out using a dilution counting technique (Merselis, Kaye, Connolly & Hook, 1964). This method was used in preference to multiple tube enrichments as used by McCall, Martin & Boring (1966) because of greater ability to cover a wide range of counts

* Both Difco and Baltimore Biological Laboratories media were used throughout these studies.

with minimal media and laboratory examinations. The sensitivity of the method used, however, does not allow estimations below 100 orgs./g. faeces. A 10% suspension of faeces was made in quarter strength Ringer solution in a container with sterile glass beads and shaken for a few minutes. Tenfold dilutions in the same diluent were then made and 0·1 ml. of appropriate dilutions spread on BG plates with glass spreaders. Duplicate plates were spread from each dilution and a count of typical salmonella colonies made after 24–36 hr. Biochemical and serological identification was performed on randomly selected colonies. Double strength tetrathionate broth was added to one dilution (10^{-2}) and any salmonellas present recorded during the period that quantitative direct plating of faeces was in progress. Where the level of excretion dropped to 100 salmonellas/g. of faeces, alginate swabs were placed directly in 10–12 ml. enrichment broth.

Post-mortem samples

Calves were destroyed by exsanguination and, after skinning, carcasses were flamed with a butane burner. Asceptic precautions were taken in the removal of specimens, but following the recommendation of Kampelmacher, Guinée & Jansen (1964) all tissues were placed in water at 100° C. for about 6 sec. to reduce superficial contamination. The following tissues were examined: mandibular, parotid, suprapharyngeal, all mesenteric, caecal, colic, prescapular and precrural lymph nodes, spleen, liver (including gall bladder and hepatic lymph node) and kidney and tonsillar tissue. In addition, contents of rumen, abomasum, small intestine, caecum and rectum were examined for salmonellas. Spleen, liver, kidney and mesenteric lymph nodes were homogenized in a domestic blender and approximately 10 g. added to 100 ml. broth. Approximately 5 g. of contents of the gastro-intestinal tract were added to 100 ml. broth. In the early stages of this experiment, the small intestine was removed and divided into three or four sections and each portion homogenized. Later, in the calves dosed with 10^5 and 10^4 organisms, the less cumbersome method used by Williams Smith (1955) was adopted, viz. small sections were taken of duodenum, jejunum, and ileum about 3 in. long, the exterior flamed, slit longitudinally and a swab of the contents taken. The piece of intestine was washed in running water followed by soaking in 70% ethyl alcohol for 1 min. and again washed in water. The intestine was cut up finely with scissors and cultured in enrichment broth.

Experimental animals

Jersey calves were obtained from the dairy farms of Ruakura Agricultural Research Centre. To determine that these herds were free from salmonella infection at the time, swabs (Moore, 1948) were placed in the main drain leading from each dairy and cultured at weekly intervals. This procedure had been used previously (Robinson, 1966) to detect the presence of salmonella-excreting cows. Rectal swabs were also taken from all calves before experimental infection. All calves were left with the adult cow for 2 days after birth. Calves to be dosed at 14–21 days were weaned at 4 days of age and then reared in strict isolation. Calves were kept out of doors singly in pens of approximately 80–100 ft.^{2} and given whole

raw milk twice daily plus hay *ad lib*. Direct contact between calves did not occur. The only potential avenue of cross-infection was via the person sampling or feeding. Milk was checked occasionally for the presence of salmonellas. All feeding equipment (rubber teats, tubes and buckets) was rinsed in cold water followed by hot water after each feed.

Thirty-eight calves were given doses of viable *S. typhimurium* as set out in Table 1. The mean age of the older calves was 18 days when dosed.

Table 1. *Schedule of doses for older and younger calves*

Approximate dose	Number of calves	
	Younger	Older
10^6	12	11
10^5	3	4
10^4	4	4

RESULTS

There appears to be little or no information available about when viable organisms could be recovered from tissues following apparent cessation of faecal excretion. Whenever possible, at least 3 weeks of negative salmonella excretion was recorded before an animal was slaughtered, but eleven calves were killed after a shorter period, and three of the younger animals after less than a week of negative excretion. Occasionally a positive sample was obtained during a long run of negatives. Since calves were kept continuously in individual pens, these isolated positives may be the result of recent ingestion of a few organisms from the environment, or of the original infection in the calf; since it is not possible to be certain on this point no account was taken of these few positives in recording time to the last positive swab.

Table 2 shows, for individual calves, the time in days from dosing to slaughter, the time in days from dosing to the last positive rectal swab recorded, and the site of recovery of organisms after slaughter.

Duration of excretion

Overall analysis, using log (days + 1) to stabilize the variance, showed ($P < 0{\cdot}01$) that the younger calves excreted salmonellas for longer than the old animals, but indicated ($P < 0{\cdot}10$) that the ratio depended on the dose, being three and eight times longer for the 10^6 and 10^5 orgs/dose groups respectively. Little weight can be given to the corresponding ratio from the 10^4 orgs./dose group, as only one of the older calves recorded a positive, and that on the first day after dosing.

Sites of recovery of salmonellas

S. typhimurium was recovered at slaughter from 10/19 of the younger calves and 4/19 of the older animals. Among the younger calves receiving 10^6 organisms, salmonellas were found in four of the five killed after less than 3 weeks negative sampling compared with three of the remaining seven. The organisms were

Table 2. *Duration of faecal excretion, and post-slaughter recovery sites, of* Salmonella typhimurium

No. of orgs. in dose	Calf	Days from dosing to: Slaughter	Days from dosing to: Last +ve swab	Recovery sites of organisms after slaughter
		Calves dosed before 3 days of age		
10^6	0064	38	25	+ (mes. L.N.)
	0057	46	46	+ (mes. L.N., ileum, rectal contents)
	0005	56	20	+ (mes. L.N., caecal contents)
	0003	63	41	–
	0024	65	44	–
	0052	81	21	+ (phary. L.N., caecal contents)
	0002	103	72	–
	0058	130	125	+ (mes. L.N.)
	0051	139	115	–
	0067	151	135	+ (mes. L.N.)
	0001	230	168	–
	0004	236	194	+ (mes. L.N.)
Geometric mean			63·4	
10^5	0091	21	0	–
	0090	34	28	–
	0196	105	77	–
Geometric mean			12·1	
10^4	0198	82	8	–
	0193	82	52	+ (mes. L.N., caecal L.N.)
	0194	105	30	+ (mes. L.N.)
	0197	112	36	+ (mes. L.N., jejunal wall)
Geometric mean			26·2	
		Calves dosed at 14–21 days of age		
10^6	0070	34	12	+ (mes. L.N.)
	0026	37	27	–
	0031	44	12	–
	0032	44	1	+ (liver, duodenum)
	0080	44	15	+ (caecal L.N.)
	0025	50	25	–
	0016	64	16	–
	0023	64	26	–
	0066	64	32	–
	0022	69	51	–
	0065	140	129	+ (mes. L.N.)
Geometric mean			20·3	
10^5	0014	21	0	–
	0100	26	0	–
	0015	30	1	–
	0099	42	15	–
Geometric mean			1·4	
10^4	0067	19	0	–
	0069	19	0	–
	0056	26	0	–
	0007	27	1	–
Geometric mean			0·2	

https://doi.org/10.1017/S0022172400041085 Published online by Cambridge University Press

recovered from the mesenteric, caecal and colic lymph nodes in twelve out of fourteen of the positive calves, indicating that in these studies the above sites appeared to be the most useful indicators of infection. This differs from the results of Guineé, Kampelmacher, van Keulen & Hofstra (1964) who considered that the mesenteric lymph nodes were less reliable indicators than the gall bladder.

Table 3. *Degree of faecal excretion of* Salmonella typhimurium *in calves dosed with approximately* 10^6 *living organisms*

Days after dosing	Excretion of *S. typhimurium* log orgs./g. Negative	< 2	$< 3{\cdot}5$	< 5	$\geqslant 5$	Estimated median concentration (orgs./g.)
	Six calves dosed before 3 days of age					
1	—	1	1	4	—	$2{\cdot}7 \times 10^3$
2	—	—	1	4	1	$1{\cdot}5 \times 10^4$
3	—	—	2	3	1	$1{\cdot}3 \times 10^4$
4	—	1	1	2	2	$6{\cdot}1 \times 10^3$
5	1	—	2	1	2	$1{\cdot}8 \times 10^4$
6	1	—	2	1	2	$2{\cdot}5 \times 10^3$
7	1	1	—	4	—	$5{\cdot}3 \times 10^3$
8	1	2	2	1	—	$2{\cdot}7 \times 10^2$
9	—	2	3	1	—	$1{\cdot}3 \times 10^2$
10	1	3	1	1	—	$4{\cdot}6 \times 10$
11	1	3	1	1	—	$0{\cdot}3 \times 10$
12	2	2	2	—	—	$1{\cdot}2 \times 10^2$
13	2	2	2	—	—	$0{\cdot}9 \times 10$
14	3	2	1	—	—	—
	Six calves dosed 14–21 days of age					
1	—	2	3	1	—	$6{\cdot}7 \times 10^2$
2	—	2	3	1	—	$1{\cdot}1 \times 10^3$
3	1	3	2	—	—	$3{\cdot}0 \times 10^2$
4	1	3	2	—	—	$6{\cdot}0 \times 10^2$
5	1	4	—	1	—	—
6	1	2	2	1	—	$1{\cdot}2 \times 10^2$
7	1	3	2	—	—	$2{\cdot}0 \times 10^3$
8	1	1	4	—	—	$2{\cdot}1 \times 10^2$
9	3	3	—	—	—	—
10	3	3	—	—	—	—
11	5	1	—	—	—	—
12	4	2	—	—	—	—
13	5	1	—	—	—	—
14	4	2	—	—	—	—

However, when these workers examined all the mesenteric lymph nodes instead of only the caudal portions, their isolation of salmonellas doubled, indicating that the organisms appeared to be distributed uniformly over the whole mesenteric nodal system. Although the gall bladder and associated section of liver and hepatic lymph node were examined in our studies, *S. typhimurium* was recovered from these sites only once. In one calf (0197) salmonellas were recovered from the wall of the jejunum but not from the jejunal contents at that point. While it is possible

that the calves still harbouring salmonellas at slaughter could have recommenced excreting them, it is considered that the presence of organisms in the mesenteric lymph nodes only, represents the terminal phase of recovery from the original *infection*.

Salmonella concentration in the faeces

One measure of the infectivity of a calf to others is the number of salmonellas it sheds in its faeces. Therefore an investigation of the number of organisms excreted per gram of faeces was carried out. Table 3 summarizes the data from six younger and six older calves dosed with 10^6 *S. typhimurium*, for the first 14 days after dosing. The geometric means shown are estimates of the median level of excretion, and were obtained, using the tables of Sarhan & Greenberg (1956), by regarding samples rated at less than 10^2 orgs./g. as censored data from the same population as the estimable concentrations. On 4 of the first 7 days after dosing there were significantly ($P < 0{\cdot}05$, single tail) more younger calves with salmonella concentrations in the faeces greater than $3{\cdot}2 \times 10^3$ orgs./g. (i.e. antilog 3·5), and their median level was higher than for older calves on all days for which both estimates were available.

Counts were also made on faeces from calves dosed at the lower dose rates but only once was a count of greater than 10^2 orgs./g. detected. Calf no. 0196 dosed with 10^5 *S. typhimurium* at 2 days of age, continued to excrete for 14 days at 10^3 salmonellas/g. faeces.

DISCUSSION

Newell (1967) has stated that *Salmonella* infections are related to four independent factors, personal susceptibility, the risk of direct or indirect contact with a serotype, the number of organisms ingested, and serotype characteristics. In these studies we have endeavoured to examine one component of the first variable only, i.e. age. It was observed (Robinson, 1966) in an earlier trial that two calves dosed with 10^6 *S. typhimurium* at 2 days of age were more infective among their peers at 4 days of age than a similarly dosed pair were at 21 days of age. In these trials eight undosed in-contact calves in each group were killed after 24 hr. close confinement and transport with the dosed animals, and salmonella organisms were isolated from seven of the young group but from none of the older calves ($P < 0{\cdot}01$). This difference could be the result of either lower excretion rate of the dosed calves after 19 days infection or lowered susceptibility of the older calves. It would appear from the present studies that both factors operate. Calves dosed at 2 days of age are likely to be excreting few organisms at 21 days of age. If we accept that the number of organisms excreted following dosing is one indicator of susceptibility to infection, then the older dosed animals appear to be more resistant. Throughout these trials it was assumed that salmonella-excreting and salmonella-free animals would have come from separate farms before congregation. If, for instance, a 21-day-old calf was exposed to salmonellas 2 or 3 days before sale it is likely that it could contribute to cross-infection.

Other workers have demonstrated similar patterns of age resistance to enteric infections. Mushin & Dubos (1965) using an enteropathogenic strain of *Escherichia*

coli in mice found that the period of greatest susceptibility to oral infection extended from the day of birth until 2 weeks of age. After this time, colonization of the gastro-intestinal tract became more difficult, even when very large doses were administered on 3 consecutive days. They suggested that development in the gastro-intestinal tract of a microbiota which is antagonistic to *E. coli* may be responsible for this acquisition of resistance with age. Szanton (1957) has shown that new born infants infected with *S. oranienburg* during an outbreak continued to excrete the organism much longer than adults infected during the same outbreak.

A rapid increase in resistance to experimental salmonellosis with age has been demonstrated in both turkeys and chickens. Bierer (1960) has shown that a 90% mortality can be expected in turkey poults given varying doses of *S. typhimurium* within the first 6 hr. after hatching. The same doses (up to 10^9 viable organisms) given to birds at 72 hr. of age produced no effects. Severens, Roberts & Card (1944) and Williams Smith (1955) have also shown that a rapid increase in resistance to oral infection with *S. pullorum* developed in chickens during the first few days of life.

Diarrhoea is common among calves during the first 3 weeks of life, and if it is associated with salmonella excretion the risk of extensive environmental contamination is obvious. In these studies most calves exhibited diarrhoea at some stage, often accompanied by reduced appetite. The diarrhoea did not appear to be related to either dosing or high faecal salmonella counts. Apart from a reduction in milk intake no therapeutic measures were considered necessary.

It is recognized that calves from which salmonellas were recovered at slaughter may have recommenced faecal excretion following the pre-slaughter negative period, but it is difficult to eliminate the possibility of continuous re-ingestion of organisms from the environment. The persistence of these pathogens in calf pens and sheds is lengthy. Adinarayahan, Smyser & Roekel (1966) have observed that poultry excreting *S. heidelberg* cleared themselves of infection within a few days of being transferred to wire batteries; whereas their pen mates maintained in the original contaminated colony house continued to excrete this serotype intermittently. In the absence of re-infection therefore it would appear that *S. typhimurium* infection in calves is self-limiting and that most calves do not remain permanent shedders of the organism.

Williams Smith (1955) claims that there was no evidence that salmonellas multiplied within the lumen of the intestine. The recovery of *S. typhimurium* from the intestinal wall but not from the contents in one calf suggests that this is likely. Recent fluorescent antibody studies by Kent, Formal & Labrec (1966) of *S. typhimurium* infection in Rhesus monkeys show the organisms present in the surface epithelial cells, lamina propria and submucosa of the small intestine as well as in the associated lymph node.

SUMMARY

Two groups of calves were dosed orally with 10^4, 10^5, and 10^6 *S. typhimurium* at approximately 2 days of age and at 14–21 days of age. No obvious clinical signs were observed with this strain, but younger calves excreted the salmonellas in the

faeces for longer periods than older animals. The younger animals also excreted more salmonellas per gram of faeces in the first week following dosing. These observations may explain why salmonella cross-infection is likely to occur where very young calves are congregated. Following cessation of excretion of salmonellas in the faeces and subsequent slaughter of calves, examination of all the mesenteric, caecal, and colic lymph nodes showed these to be the most useful sites for recovery of salmonellas.

Provided good nutrition and hygienic conditions prevail, calves retained on their farm of origin for longer periods are more likely to recover from neonatal salmonella infections.

We wish to acknowledge the capable laboratory assistance of Miss R. Allen, B.Sc., Miss H. MacDiarmid, Mrs C. Clark, Miss V. Filby and Miss J. Herrold. We also thank Mr D. C. Westwood for care of animals and Mr D. M. Duganzich for statistical assistance.

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