The Iron Milk Most Probable Number Method for Enumeration of *Clostridium perfringens* in the Diet and the Intestine of the Chick

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ABSTRACT Seven experiments were conducted to evaluate the iron milk most probable number method for enumeration of *Clostridium perfringens* in the diet and the intestine of broiler chicks. Levels of 50 ppm of neomycin and 20 ppm of polymixin improved the iron milk tube method when compared to the TSN (tryptone-sulfite-neomycin) agar plate method. Low numbers of the organism, approximately 5 per gram, were detected in the practical diet fed to the chicks. Vegetative cell numbers of *C. perfringens* increased from 1.7 $\log_{10}$ in the duodenum of chicks to >9.2 $\log_{10}$ in the ceca. Spores of the organism were detected in the ileum and ceca. Results of two experiments demonstrated that *C. perfringens* became established in the ileum of chicks early in life, before initiation of feeding at 2 days of age.

(Key words: Clostridium perfringens, iron milk most probable number method, intestine, diet, chicks)

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INTRODUCTION

Clostridium perfringens has been implicated in the depression of growth of monogastric animals since the advent of antibiotic usage in the 1950's. This anaerobic organism has also been shown to be sensitive *in vitro* to nearly all known feed additive antimicrobial agents in use today for poultry and swine. Investigations of the organism *in vivo,* however, have been limited because of convenient and repeatable techniques for enumeration of the organism.

Several plating methods, developed for evaluation of foods, have been utilized to determine the number of clostridia in the intestine of avian species. Wagner and Wostmann (1959) used a modified sulfite iron agar method of Mossel *et al.* (1956) to determine the number of clostridia in the ileum of White Leghorn chickens. The method was not specific for *C. perfringens.* Smith (1965) determined the numbers of *C. perfringens* in various sections of the intestinal tract of chickens and ducks using the neomycin Nagler agar method of Lowbury and Lilly (1955). Timms (1968) employed the RCM (reinforced clostridial medium) agar method of Hirsch and Grinsted (1954) with added blood and neomycin to determine the numbers of *C. perfringens* in the small intestine and ceca of three age groups of chickens. Naqi *et al.* (1970) utilized the SPS (sulfite-polymixin-sulfadiazine) agar method of Angelotti *et al.* (1962) to estimate *Clostridium spp.* in the small and large intestine of turkey poults at various ages. The TSN (tryptone-sulfite-neomycin) agar plate method of Marshall *et al.* (1965) was employed by Stutz *et al.* (1983a,b,c) to evaluate the effects of antibiotics on *C. perfringens* numbers in the ileum of broiler chicks.

Tube methods are generally less time-consuming and less tedious than plating methods and do not require the additional equipment to maintain anaerobic conditions. Smyser *et al.* (1952) estimated *C. perfringens* numbers in the feces of broiler chickens by determining the most probable number (MPN) using the iron skin milk medium of Spray (1936). Erickson and Deibel (1978) reported on a new MPN tube method for determination of *C. perfringens* in foods using a litmus milk and gelatin-based media with added neomycin and polymixin. The method was shown to be more sensitive than plating methods, but the method requires considerable time to prepare the complex media. Wekell *et al.* (1980) and St. John *et al.* (1982) developed a simple iron milk MPN tube assay for enumeration of *C. perfringens* in the intestinal tract of the rat and in soil, sludge, and water samples. The media contained no antibiotics and positive tubes were determined by stormy fermentation after incubation at 45 C for 16 to 24 hr.

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The objectives of the following experiments were to evaluate the iron milk most probable number method for enumeration of *C. perfringens* in the diet and the intestine of the broiler chick. Investigations were conducted to determine the effects of media and antibiotics on *C. perfringens* numbers, to compare the iron milk tube method with the TSN agar plate method, and to determine numbers of vegetative cells and spores of *C. perfringens* in various sections of the intestinal tract of the chick. The establishment of the organism in the ileum of the chick was also investigated.

MATERIALS AND METHODS

The chicks utilized in four of the experiments were controls from growth and feed efficiency studies that consisted of 24 pens of eight chicks each with six replicates of controls per experiment. Two-day-old male broiler-cross chicks (Hubbard × Hubbard) were started on a nonmedicated practical diet (milled Laboratory Chick Chow #5065, Ralston Purina Company, St. Louis, MO). The practical corn and soybean meal-based diet contained not less than 21% protein and 3% fat. For Experiments 1, 3, 4, and 5 the chicks were grouped on a weight basis at 3 days of age and fed practical experimental diets for an 8-day period. At termination of these experiments, control chicks were maintained on the practical diet and sacrificed for intestinal contents for the following 4 days (12 to 15 days of age). For Experiment 6, the chicks were grouped on a weight basis at 3 days of age, fed the practical diet, and sacrificed for the ileum and its contents and ileal contents at days 1 to 3 and 5 to 9, respectively (3 to 11 days of age). The chicks were maintained in battery brooders with raised wire floors and subjected to continuous lighting. Feed and water were supplied *ad libitum* in all of the experiments except in Experiments 6 and 7 where two groups of 6 chicks each were sacrificed for the ileum and contents before initiation of feeding and watering at 2 days of age. For Experiment 2, six samples of the practical corn and soybean meal diet fed to the chicks in the other experiments were retained for enumeration of *C. perfringens*.

Procedures used for collection and dilution of samples for determinations of *C. perfringens* numbers were similar to those of Wekell *et al.* (1980). Approximately 1-g samples of intestinal contents or feed were transferred into tared test tubes containing 9 ml of sterile .1% peptone (Difco). The peptone solution was steamed within 30 min before use and was held at 46 C in a water bath. In those experiments where the ileum and contents were utilized, the total weight of the ileum and contents of each chick were homogenized in sterile .1% peptone at a ratio of 1:9. All samples were mixed for 5 sec with a Vortex mixer and serially diluted using sterile .1% peptone.

The iron milk 3-tube MPN method of Wekell *et al.* (1980) and St. John *et al.* (1982), with minor modifications, was used for enumeration of *C. perfringens* in the samples. Iron milk media was prepared by adding 10 ml of whole milk to approximately .2 g of iron powder in 125 × 20-mm screw cap culture tubes. The tubed media were steamed for 5 min and maintained in a water bath at 46 C. One milliliter aliquots of the serial dilutions of the samples were immediately inoculated into the iron milk and the tubes were transferred to an incubator. Positive tubes were determined by stormy fermentation after incubation at 46 C for approximately 21 hr. For the antibiotic supplemented iron milk media 50 ppm of neomycin, as neomycin sulfate, and 20 ppm of polymixin, as polymixin B sulfate, were added to the whole milk just prior to dispensing. Spore counts were determined by heating diluted samples to 80 C for 5 min and enumerating as for vegetative cells.

The TSN agar plate method of Marshall *et al.* (1965) was utilized to confirm anaerobic growth and sulfite reduction of positive iron milk tubes and to compare the tube method to a plating method for enumeration of *C. perfringens*. Procedures for the TSN agar plate method were similar to those described by Stutz *et al.* (1983a,b,c) One-half milliliter of the serial dilutions or the contents of incubated iron milk tubes were spread on agar plates followed by TSN agar (BBL) overlays. The plates were incubated anaerobically at 46 C for approximately 24 hr using the Gas Pak system (BBL).

Bacteria determined by the methods are reported as organisms per gram and were analyzed for significance using the Mann-Whitney U test (Campbell, 1974).

RESULTS

The effects of media and antibiotics on the determination of *C. perfringens* in the ileum of

broiler chicks are presented in Table 1. Addition of the antibiotics neomycin and polymixin to the iron milk media resulted in numbers of the organism similar to those confirmed by the agar plate method. The levels of the antibiotics used in the iron milk media, 50 ppm of neomycin and 20 ppm of polymixin, were similar to those present in TSN agar plate media. The absence of antibiotics in the iron milk media resulted in a significant overestimation of *C. perfringens* numbers and indicated that other competing organisms were likely present.

Table 2 depicts the effects of media and antibiotics on the determination of *C. perfringens* in the practical chick diet. Results were similar to those in the ileum in that the iron milk media without antibiotics detected significantly higher numbers of organisms. The results also indicate that the diet may be the origin of the competing organisms in the intestine of the chick.

A comparison of the iron milk tube method, supplemented with antibiotics, with the TSN agar plate method for the determination of *C. perfringens* in the ileum of broiler chicks is presented in Table 3. No significant difference was observed between the two methods in the numbers of the organism detected.

Clostridium perfringens counts in the three sections of the small intestine of broiler chicks, determined by the modified iron milk method, are presented in Table 4. The numbers of the organism significantly increased from the duodenum to the ileum.

Table 5 depicts the numbers of vegetative cells and spores of *C. perfringens* in the ileum and ceca of broiler chicks determined by the modified iron milk method. The numbers of both vegetative cells and spores were significantly higher in the ceca when compared to the ileum.

The establishment of *C. perfringens* in the ileum of broiler chicks is presented in Tables 6

TABLE 1. *Effects of media and antibiotics on the determination of Clostridium perfringens in the intestine of broiler chicks (Experiment 1)*

Method	Numbers in ileal contents	
	($\log_{10}$/g)	
Iron milk tube	>5.8[a]	(3.3 – >6.7)[3]
TSN agar plate[1]	4.2[b]	(2.6 – 6.7)
Iron milk tube and antibiotics[2]	3.9[b]	(2.5 – 6.7)
TSN agar plate[1]	3.9[b]	(2.6 – 6.7)

[a,b]Means with different superscript letters are significantly different (P<.05).

[1] Positive tubes confirmed by agar plate method. TSN = Tryptone-sulfite-neomycin.

[2] Media supplemented with 50 ppm of neomycin and 20 ppm of polymixin.

[3] Values in parentheses represent the ranges for nine chicks for the four methods.

TABLE 2. *Effects of media and antibiotics on the determination of Clostridium perfringens in the practical chick diet (Experiment 2)*

Method	Numbers in diet	
	($\log_{10}$/g)	
Iron milk tube	3.9[a]	(3.4 – 4.3)[3]
TSN agar plate[1]	.7[b]	(0 – 1.1)
Iron milk tube and antibiotics[2]	.4[b]	(0 – .9)

[a,b]Means with different superscript letters are significantly different (P<.05).

[1] Positive tubes confirmed by agar plate method. TSN = Tryptone-sulfite-neomycin.

[2] Media supplemented with 50 ppm of neomycin and 20 ppm of polymixin.

[3] Values in parentheses represent the ranges for six diet samples for the three methods.

TABLE 3. *Comparison of the iron milk tube method with the tryptone-sulfite-neomycin (TSN) agar plate method for the determination of Clostridium perfringens in the intestine of broiler chicks (Experiment 3).*

Method	Numbers in ileal contents	
	($\log_{10}$/g)	
Iron milk tube[1]	3.1[a]	(1.6 – 5.4)[2]
TSN agar plate	3.3[a]	(2.2 – 6.8)

[a]Means with the same superscript letter are not significantly different (P>.05).

[1] Media supplemented with 50 ppm of neomycin and 20 ppm of polymixin.

[2] Values in parentheses represent the ranges for 12 chicks for the two methods.

TABLE 4. *Clostridium perfringens counts in the small intestine of broiler chicks determined by the iron milk tube method with added antibiotics (Experiment 4)*

Intestinal section	Numbers in sectional contents	
	($\log_{10}$/g)	
Duodenum	1.7[a]	(1.4 – 2.6)[1]
Jejunum	2.4[b]	(1.6 – 3.4)
Ileum	3.0[c]	(2.4 – 5.0)

[a,b,c]Means with different superscript letters are significantly different (P<.05).

[1] Values in parentheses represent the ranges for eight chicks for the three sections of the small intestine.

and 7 (Experiments 6 and 7). Numbers of the organism determined by the iron milk method supplemented with antibiotics, in the ileum and contents of chicks before feeding, indicated that there was no significant difference between the two experiments. In Experiment 6 (Tables 6 and 7), *C. perfringens* numbers did not significantly change from 1 to 3 days or 5 to 9 days after initiation of feeding (3 to 11 days of age). The results of these experiments demonstrate that the organism becomes established in the intestine of the chick early in life, before initiation of feeding.

DISCUSSION

The results of Experiments 1 and 2 demonstrated that the iron milk tube method, without added antibiotics, overestimated the *C. perfringens* numbers in both the ileum and the diet of chicks. One explanation is that other competing organisms were present that produced a stormy fermentation similar to that of *C. perfringens.* Smyser *et al.* (1952) isolated a bacillus from positive iron skim milk tubes that possessed the characteristics of *C. perfringens.* St. John *et al.* (1982) reported that both bacillus and coliforms produced reactions similar to stormy fermentation, but that their reactions were slower than for *C. perfringens* at 45 C. Nearly all media for the enumeration of *C. perfringens* contain antibiotics to selectively inhibit competing facultative organisms. The addition of neomycin and polymixin to the iron milk media significantly improved the method, especially for determinations of the organism in the intestine and the diet of the chick as described here. A comparison of the modified tube method with the TSN agar plate method of Marshall *et al.* (1965) indicated excellent agreement for determination of *C. perfringens* in the ileum of chicks. These values were similar to those previously reported by Stutz *et al.* (1983b) for chicks fed a practical diet and the TSN agar plate method was employed.

Clostridium perfringens numbers increased from the duodenum to the ceca and spores of the organism were detected in the ileum and ceca of chicks fed the practical diet (Experi-

TABLE 5. *Vegetative cells and spores of Clostridium perfringens in the intestine of broiler chicks determined by the iron milk tube method with added antibiotics (Experiment 5)*

Section	$\log_{10}$ counts per gram sectional contents	
	Vegetative cells	Spores
Ileum	2.8[a] (2.4– 4.0)[1]	<1.7[a] (<1.5–2.0)
Ceca	>9.2[b] (8.4–>9.4)	3.3[b] (1.6–4.3)

[a,b]Means within a column with different superscript letters are significantly different (P<.01).

[1] Values in parentheses represent the ranges for six chicks for the two intestinal sections.

TABLE 6. *Establishment of Clostridium perfringens in the intestine of broiler chicks from before feeding to 3 days after feeding*

Day[1]	Numbers in ileum and contents	
	($\log_{10}$/g)	
Experiment 6		
0	3.8[ab]	(2.5 – 4.7)[2]
1	4.0[b]	(2.4 – 5.2)
2	4.6[b]	(4.0 – 5.4)
3	3.5[ab]	(2.5 – 5.3)
Experiment 7		
0	2.9[a]	(2.5 – 3.3)

[a,b]Means with different superscript letters are significantly different (P<.05).

[1] Day 0 represents before initiation of feeding at 2 days of age.

[2] Values in parentheses represent the ranges for six chicks for each day.

TABLE 7. *Clostridium perfringens counts in the intestine of broiler chicks from 5 to 9 days after initiation of feeding*

Day[1]	Numbers in ileal contents	
	($\log_{10}$/g)	
Experiment 6		
5	4.7[a]	(3.0 – 5.4)[2]
6	3.7[a]	(2.6 – 5.2)
7	3.8[a]	(2.6 – 5.2)
8	4.3[a]	(2.9 – 5.4)
9	3.4[a]	(2.4 – 5.4)

[a]Means with the same superscript letter are not significantly different ($P<.05$).

[1] Days 5 to 9 represent 7 to 11 days of age.

[2] Values in parentheses represent the ranges for six chicks for each day.

ments 4 and 5). Both Smith (1965) and Timms (1968) were unable to detect significant numbers of *C. perfringens* in the small intestine of chickens but did observe low levels of the organism in the ceca. Wagner and Wostmann (1959) obtained high numbers for clostridia in the ileum of chickens fed a practical diet. The method, however, was not specific for *C. perfringens*, and other species of clostridia may have been detected. Our results agree with those of Lev *et al.* (1957) and Barnes *et al.* (1980) that high numbers of *C. perfringens* are found in the ceca of chicks fed a practical diet. Lev *et al.* (1957) also detected spores of the organism in the ceca of chicks. Surprisingly few reports exist in the literature that relate to vegetative and spore numbers of *C. perfringens* in the intestine of chickens.

The results of two experiments demonstrated that *C. perfringens* became established in the intestine of the chick early in life, before initiation of feeding at 2 days of age. Lev *et al.* (1957) and Naqi *et al.* (1970) reported that *C. perfringens* and *Clostridium spp.* became established in the intestine of chicks and turkey poults, respectively, 1 day after feeding. Barnes *et al.* (1980) observed high numbers of microorganisms, including *Clostridium spp.*, in the ceca of newly hatched chicks from commercial hatcheries before they were fed. They suggested that the chicks may have developed the flora while still in the hatchers or from the boxes in which they were transported. Further research is needed to determine the origin and the establishment of the organism *C. perfringens* in the intestine of the newly hatched chick.

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