# A sensitive method for isolating *Fusobacterium necrophorum* from faeces

G. R. SMITH, S. A. BARTON AND L. M. WALLACE

*Institute of Zoology, The Zoological Society of London, Regent's Park,
London NW1 4RY*

## SUMMARY

The isolation of *Fusobacterium necrophorum* present in small numbers in heavily contaminated material such as faeces or soil is hampered by the lack of an efficient selective medium and by the high minimum infective dose of the organism. A sensitive method for the detection and isolation of faecal strains of *F. necrophorum* type A was based on the subcutaneous injection of faeces, suspended (5 % w/v) in broth culture of *Actinomyces* (*Corynebacterium*) *pyogenes* or *Staphylococcus aureus* to increase fusobacterial infectivity, into mice pretreated with clostridial antitoxins. When necrobacillosis developed *F. necrophorum* was identified microscopically in tissue from the advancing edge of the lesion and isolated on a partly selective medium.

The enhancement of fusobacterial infectivity produced by *A. pyogenes* and by *S. aureus* was high, but the latter was slightly the more efficient, enabling as few as 80 *F. necrophorum* organisms/g of faeces to be detected.

Use of the method showed that 3 of 16 wallabies had *F. necrophorum* in their faeces at the time of examination. Numerous epidemiological applications are suggested.

## INTRODUCTION

Necrobacillosis is a common disease of animals, occurring in a wide range of domesticated and wild (free-living and captive) species. The lesions (coagulative necrosis) occur in a variety of sites, but the mouth, liver and foot are among the most frequently affected in cattle and in other herbivores such as deer and antelope [1]. The disease is the commonest affliction of macropods in captivity. Necrobacillosis lesions usually contain not only the causative organism, *Fusobacterium necrophorum*, but also a mixture of other bacteria. *F. necrophorum* strains of phase A [2] are haemolytic, haemagglutinating, leucocidinogenic and pathogenic for mice; phase B strains are non-haemagglutinating and have no more than slight pathogenicity. Phase (type, biovar) A organisms are found in necrobacillosis lesions but in the gut contents of cattle are outnumbered by phase B. Nonetheless necrobacillosis of the external body surface and mouth is thought to result from faecal contamination of small wounds and abrasions with type A strains.

There have been a number of attempts to devise selective techniques for the isolation of *F. necrophorum* from contaminated materials [3–5]. Little is known,

however, of the frequency with which type A strains are excreted in animal faeces, of the factors influencing such excretion, or of the survival of the organism in faecal deposits. This is because of (a) the lack of a truly efficient selective medium for *F. necrophorum*, and (b) the high minimum infective dose ($> 10^6$) of the organism for laboratory animals such as mice by subcutaneous inoculation.

It has recently been shown [6, 7] that the presence of certain organisms such as *Escherichia coli, Citrobacter freundii, Actinomyces (Corynebacterium) pyogenes, Staphylococcus aureus, Klebsiella oxytoca* or *Bacillus cereus* strongly enhances the infectivity of *F. necrophorum* for mice by subcutaneous inoculation. Suspension of *F. necrophorum* in sub-lethal doses of cultures of such organisms may thus reduce the minimum infective dose by a factor of $c.\ 10^6$.

It might be thought therefore that the subcutaneous inoculation of mice with faecal suspension would be a sensitive indicator of *F. necrophorum* in the faeces – by virtue of the infectivity-enhancing properties of the mixed faecal microflora. Support for this notion was reported earlier [8]. Subsequent studies on a larger number of samples have shown, however, that the use of faecal suspensions diluted to 5 % w/v to avoid unworkably dense inocula and unacceptably high numbers of non-specific deaths reduces the concentration of faecal organisms to a point at which enhancement of fusobacterial infectivity will sometimes but by no means always occur.

The present paper describes an *in vivo* method, based on suspending faeces in cultures of *A. pyogenes* or *S. aureus* to increase fusobacterial infectivity, by means of which minimal numbers of *F. necrophorum* type A organisms can be demonstrated in faeces and isolated for further study. The method will be used to investigate factors concerned in the spread of necrobacillosis, a disease that causes much suffering and economic loss in animals.

## MATERIALS AND METHODS

The culture media, anaerobic methods and viable count technique were as already described [9] unless otherwise stated.

### Organisms

The strains of *F. necrophorum* (A42), *Actinomyces (Corynebacterium) pyogenes* and *Staphylococcus aureus* have been described previously [7].

### Mice

Female Swiss white mice weighing 20–22 g were obtained from an outbred closed colony.

### Suspensions (homogenates) of gaur (Bos gaurus) and wallaby (Macropus rufogriseus) faeces

Gaur faeces was homogenized within 1 h of being passed. Wallaby faeces was obtained from dead animals at routine necropsy, specimens often being submitted as a tied-off section of rectum; such specimens were examined the same day or after overnight refrigeration. Each faecal sample was homogenized either in a 24 h BM broth [10] culture of *A. pyogenes* or *S. aureus* diluted 1 in 2·5 with sterile

medium, or in sterile BM medium, to form a 5% w/v suspension. Gross faecal debris was allowed to settle and the supernate was used to inoculate mice.

*Two experiments in which gaur faecal suspensions were deliberately contaminated with known numbers of* F. necrophorum

All mice inoculated with faecal suspension were pretreated subcutaneously in the left thigh with a mixture of 0·025 ml (12·5 units) of Tetanus Antitoxin (Wellcome) and 0·075 ml of Mixed Gas Gangrene Antitoxin (Lister Institute, Elstree).

In a first experiment (Table 1) groups of three mice were inoculated subcutaneously on the outer aspect of the right thigh with 0·25 ml doses of decimal dilutions of a 24 h BM culture of *F. necrophorum* prepared in diluents consisting of faeces (initially free from *F. necrophorum*) suspended in (a) *A. pyogenes* culture, (b) *S. aureus* culture, or (c) BM medium (controls). Three additional groups of appropriate controls were included.

In a second experiment (Table 2) 0·1 ml volumes of decimal dilutions ($10^4$–$10^9$; in BM diluent) of a 24 h BM culture of *F. necrophorum* were added to bottles containing 4·9 ml volumes of faeces (again, initially free from *F. necrophorum*) suspended in (a) *A. pyogenes* culture, or (b) *S. aureus* culture. Groups of eight mice were inoculated as described above with material from each bottle. In this experiment the controls were limited to the single group needed to prove that the original sample of gaur faeces did not contain *F. necrophorum*.

*Examination of wallaby faeces for* F. necrophorum

Of samples obtained from 30 wallabies, 14 were suspended in BM medium and 16 in a BM culture of either *A. pyogenes* or *S. aureus* before being injected subcutaneously into three mice pretreated with clostridial antitoxins (see above).

*Detection of necrobacillosis in inoculated mice and isolation of* F. necrophorum *from the lesions*

The first sign of necrobacillosis was lameness, usually appearing 1–3 days after inoculation and soon accompanied by general signs of illness including ruffled fur and progressive loss of weight. To prevent suffering, affected mice were killed, usually 4–7 days after inoculation. After depilation the right hind leg with part of the flank was removed. Necrobacillosis was usually easily recognized as greyish tissue [6] spreading outward from the site of inoculation, which was frequently ulcerating. A minority of samples produced severe infections due to faecal organisms other than *F. necrophorum*. With experience such infections could usually be distinguished from necrobacillosis on clinical and pathological grounds, confirmation being provided by negative results in the microscopical and cultural examinations described below.

The leg was immersed for 2–3 sec in boiling water and diseased tissue, taken aseptically from the periphery of the lesion, was examined (a) microscopically, in impression smears stained by the rapid Giemsa method [11], for the typical beaded filaments of *F. necrophorum*, and (b) by anaerobic culture for 2 or preferably 3 days on Columbia Blood Agar (Oxoid CM331) plates containing defibrinated horse blood (Oxoid SR50) 5% and reconstituted G-N Anaerobe Supplement (Oxoid

Table 1. *Demonstration of minimal numbers of* F. necrophorum *in a suspension of faeces*

| | | Necrobacillosis in groups of three mice given FN diluted in | | |
| | | a suspension (5%) of faeces in | | |
| Dose: 0·25 ml of FN diluted 1 in | BM broth (controls) | AP* | SA* | BM broth (controls) |
|---|---|---|---|---|
| $10^2$ | 0† | N | N | 2 |
| $10^3$ | 0† | N | N | 0 |
| $10^4$ | N | 3 | 3 | 0 |
| $10^5$ | N | 3 | 3 | 0 |
| $10^6$‡ | N | 2 | 3 | 0 |
| $10^7$ | N | 0 | 2 | 0 |
| $10^8$ | N | 0 | 0 | 0 |

FN, AP and SA, *F. necrophorum*, *A. pyogenes* and *S. aureus* culture respectively.
N, not done.
\* Cultures diluted 1 in 2·5 in BM broth.
† Group of six mice.
‡ Dose contained seven *F. necrophorum* organisms.
Additional controls: 6 mice received 0·25 ml of suspension (5%) of faeces in AP (diluted 1 in 2·5) and 6 were similarly treated with a suspension of faeces in SA; all survived with no more than trivial lesions.

Table 2. *Comparison of the efficiency of* A. pyogenes *and* S. aureus *in the detection of minimal numbers of* F. necrophorum *in faeces*

| Number of F. necrophorum in dose (0·25 ml) | Deaths* (and cases of proved necrobacillosis) in groups of eight mice given FN dilutions added to a 5% homogenate of faeces in | |
| | AP† | SA† |
|---|---|---|
| 500 | 8 (8) | 7 (7) |
| 50 | 6 (6) | 8 (8) |
| 5 | 5 (3) | 7 (7) |
| 0 | 2 (0) | 4 (2) |
| 0 | 1 (0) | 2 (0) |
| 0 | 0 | 1 (0) |

FN, AP and SA, *F. necrophorum*, *A. pyogenes* and *S. aureus* culture respectively.
\* Including mice killed to prevent suffering.
† Cultures diluted 1 in 2·5 in BM broth.
Controls: eight mice that received 0·25 ml of a suspension (5%) of faeces in SA (diluted 1 in 2·5) survived with no more than trivial lesions.

SR108) 2%. On this partly selective medium positive specimens yielded large numbers of strongly haemolytic colonies which could readily be picked off and identified as *F. necrophorum* by the demonstration of Gram-negative filaments. In the examination of samples other than those deliberately contaminated with *F. necrophorum*, further confirmation of identity was obtained by isolating the organism in pure culture and subjecting it to a full range of bacteriological tests, including the production of necrobacillosis in mice by the subcutaneous injection of 0·1 ml of a 24 h culture in BM medium.

RESULTS

*Demonstration of minimal numbers of* F. necrophorum *in a suspension of faeces*

The experiment recorded in Table 1 showed that the subcutaneous inoculation of mice with gaur faeces homogenized in *A. pyogenes* or *S. aureus* culture and deliberately contaminated with minimal numbers of *F. necrophorum* was a suitable means of detecting and recovering the fusobacteria. The success of the method depended on the striking enhancement of fusobacterial infectivity produced by the presence of *A. pyogenes* or *S. aureus*. Appropriate controls showed that a 5% faecal suspension in BM broth also enhanced fusobacterial infectivity – though ·very slightly, probably because the concentration of faecal organisms had been reduced to an almost ineffective level by dilution.

*Comparison of the efficiency of* A. pyogenes *and* S. aureus *in the detection of minimal numbers of* F. necrophorum *in faeces*

Once again a sample of gaur faeces was used. Table 2 shows that *S. aureus* was probably slightly more efficient than *A. pyogenes* in enhancing the infectivity of small numbers of added *F. necrophorum*. Thus there was a statistically [12] significant difference ($P < 0.05$) between the number of cases of proved necrobacillosis produced in the presence of the two organisms when the dose of *F. necrophorum* was only five bacterial cells.

The experiment illustrated a further point of practical importance. The gaur faecal suspension itself – unlike that used in the previous experiment – produced non-specific deaths in a small proportion (*c.* 14%) of inoculated mice. As it happened, this did not affect the single control group of eight mice (included to prove that the original faeces sample did not contain *F. necrophorum*) but was revealed by most of the groups in Table 2, other than those given 50 or more *F. necrophorum* organisms/mouse. Although a few of the animals given 50 or more fusobacteria almost certainly died or were killed as a result of non-specific infections, they were nonetheless shown also to have necrobacillosis. Microscopical and cultural examination readily identified mice in other groups that died from non-specific infection in the absence of necrobacillosis.

*Examination of wallaby faeces for* F. necrophorum

Fourteen samples suspended in BM broth gave negative results. Three of 16 samples suspended in *A. pyogenes* or *S. aureus* culture were shown to contain *F. necrophorum*.

DISCUSSION

This report describes a sensitive method for the detection and isolation of *F. necrophorum* type A organisms present in faeces. The method was based on the use of bacterial broth cultures as diluent, instead of broth medium or buffer, in the preparation of 5% w/v suspensions of faeces for mouse inoculation. This in turn was based on the earlier observation [6, 7] that the presence of certain bacteria greatly enhanced the infectivity of *F. necrophorum*, an organism with an otherwise high minimum infective dose. The bacteria chosen for the present experiments were strains of *S. aureus* and *A. pyogenes*, both of which appeared to affect all

virulent strains of *F. necrophorum* equally [7] and neither of which produced by itself more than a localized lesion at the site of inoculation; such lesions sometimes ulcerated but did not lead to general signs of illness. Other potentially suitable bacteria were rejected for various reasons. For example, *E. coli* increased the infectivity of most but not all *F. necrophorum* strains [7] and occasionally caused unexpected lethal infections, especially in immature mice (unpublished); and *Bacillus cereus*, although a powerful enhancer of infectivity, caused undesirably severe ulceration in a proportion of animals [7].

Both *S. aureus* and *A. pyogenes* enabled *F. necrophorum* to be detected when it was present at a concentration of only five organisms/mouse sample. However, *S. aureus* appeared to be slightly more efficient than *A. pyogenes* and made possible the detection of even a single *F. necrophorum* organism in the inoculum (0·25 ml of a 5% faecal suspension). This was equivalent to the detection of 80 *F. necrophorum* organisms/g of faeces, which therefore represented the maximum sensitivity of the method.

Despite the pretreatment of mice with clostridial antitoxins a minority of faecal samples injected subcutaneously as a 5% w/v suspension produced lethal infections due to organisms other than *F. necrophorum*, though usually not in all of a group of three animals [8]. In the present study necrobacillosis was sometimes but not always present in mice dying from non-specific infections (Table 2); in such mice the presence or absence of the disease could always be proved by the methods of examination used. The only circumstance under which *F. necrophorum* seems likely to be overlooked is the unusual one in which death from a non-specific infection occurs so quickly that necrobacillosis has not had time to develop to a detectable extent.

It would seem, therefore, that the method described represents a suitable means of detecting *F. necrophorum* in faeces, litter or soil and of isolating the organism, even when present in only minimal numbers. This opens the way to new epidemiological investigations. The present study showed, for example, that only 3 of 16 wallabies had *F. necrophorum* in their faeces at the time of sampling. Many questions arise. What proportion of animals of various types or species excrete type A organisms in their faeces? Is excretion intermittent or do particular individuals continuously contaminate the environment? Is excretion age-related? In what numbers does the organism occur in faeces? How long does it persist in manure, litter, mud and soil, and is there ever evidence of multiplication? Answers to such questions would assist in the control of necrobacillosis in zoos and wildlife refuges, and on farms.

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