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# Wound infection: a controlled clinical and experimental demonstration of synergy between aerobic (Escherichia coli) and anaerobic (Bacteroides fragilis) bacteria

M J Kelly MChir FRCS MRCP
*Senior Surgical Registrar, Bristol Royal Infirmary*

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## Summary

*Pathogenic synergy between Escherichia coli and Bacteroides fragilis was suggested by clinical trials and proved to exist in an experimental guinea-pig wound model. This finding is thought to have important theoretical and practical therapeutic implications.*

## Introduction

It is widely recognised today among surgeons that anaerobic Gram-negative organisms of the *Bacteroides* species are deeply implicated and probably causal in the majority of infections involving abdominal wounds.

Many recent reports have unequivocally demonstrated the frequent presence of *Bacteroides* in pus taken from established wound infections *(1–5)*. Furthermore, the advent of a specific selective antianaerobic drug, metronidazole, having no activity at all against aerobes (for example, *Escherichia coli*) has dramatically reduced the sepsis rate after abdominal surgery *(6,7)*. However, demonstration of Koch's third postulate, inoculation of the subcultured *Bacteroides* into an animal of the same species to cause the same disease, has hitherto proved to be impossible.

Over the years many researchers have been totally unable to provoke any lesion by injection of cultures of *Bacteroides* into guinea-pigs, mice, rabbits, and other experimental animals *(8–10)* and doubts have arisen as to whether *Bacteroides* really is a true pathogen or merely an opportunist organism. Recently Nielsen *(11)* reported an increased incidence of liver sepsis after intravenous inoculation of already jaundiced rabbits with large doses of *B. fragilis* and that this sepsis rate was further increased by the addition of *E. coli* to the inoculum.

John Hunter, I feel sure, would have urged us to reassess the clinical *evidence* for the importance of *Bacteroides* in surgical sepsis and then to work harder at producing an experimental model that fulfilled Koch's third postulate and consequently explained the manifest success of metronidazole. This lecture reports precisely such a study.

The study fell into three phases *(12)*. In the first phase clinical trials on operative wound swabs from some 200 abdominal wounds showed a clinical association between the isolation of mixtures of aerobic and anaerobic organisms and the later development of wound infection; the second phase was the development of a reliable cheap counting method to assay such a mixture of standard bacteria in vitro; and the third phase was to test the synergy hypothesis in a quantitative experimental guinea-pig wound model.

## Clinical trials

Clinical trials were undertaken to assess the importance of different factors affecting the isolation of bacteria from operative peritoneal wound swabs. These factors included the use of transport medium, different swab material, site swabbed, and single or multiple operator laboratory processing. Wound infection was assessed by myself using the demanding criteria of Ljundqvist *(13)*.

The standard hospital system then extant

From a Hunterian Lecture delivered on 20th April 1979

*The two systems*

| *Hospital system* | *Research system* |
|---|---|
| Cotton-wool swab | Gel-foam swab |
| ↓ | ↓ |
| 'Dry' in test-tube | Stuart's transport medium |
| ↓ | ↓ |
| Kept in theatre at room temperature | + 4°C fridge |
| ↓ | ↓ |
| Hospital porters | Taken daily by author |
| ↓ | ↓ |
| Hospital microbiological laboratory Hills Road, Cambridge | Research laboratory Department of Pathology, Tennis Court Road, Cambridge |
| ↓ | ↓ |
| Multiple technician processing | Single person processing |

at Addenbrooke's Hospital, Cambridge, in 1976 was left unaltered. With the full co-operation of the Bacteriology Department, a 'research system' was set up in a separate laboratory to include as many 'improvements' as could reasonably be devised (see table). Duplicate swabs were sent through the two systems and the results were recorded separately and only correlated at the end of the trial.

A total of 214 patients were included in three groups comprising all the general surgical transperitoneal operations performed at Addenbrooke's during the period and the results were reported in considerable detail by Dr R E Warren, consultant bacteriologist, and myself in 1978 *(14)*.

The 'research system' was shown to be three to four times more effective than the 'hospital system' in providing improved isolation rates for both aerobic and anaerobic organisms and also when these results were used to predict later wound infection. The critical factor was found to be the substitution of transport medium for 'dry' swab carriage, and we recommended that all operative swabs should be sent to the bacteriological laboratory in Stuart's transport medium *(14)*.

The vast majority of those wounds that later became infected yielded positive operative wound swabs, to the extent that when a negative swab was obtained only 1% later became infected.

Even when transport medium had been used for swab carriage, naturally only some of the wounds yielded positive swabs, and of these 19/51 (37%) later developed wound infections. But in this whole group with positive swabs there were clear correlations between the cultural types (aerobic, anaerobic) of the bacteria grown and the incidence of later wound infection.

Other factors were examined and found not to have an independent association with wound infection: extent of operative contamination; type of operation; site swabbed; and antibiotic therapy. This last is an important complex element in all wound sepsis trials. We concluded that in our swab trials antibiotic treatment (which in 1976 was used in only one-fifth of the patients, and only a handful received drugs active against anaerobic organisms) had not affected our results.

Using transport medium the correlation between later wound infection and operative swab growth was:

| | |
|---|---|
| Swab sterile | 1% infected |
| Anaerobes alone | 13% „ |
| Aerobes alone | 22% „ |
| Mixed aerobes and anaerobes | 71% „ |

Thus the operative isolation of a mixed aerobic and anaerobic bacterial growth identified a

subgroup in this trial (16/122) with a 71% (11/16) incidence of later wound infection. This had potential therapeutic value in that these patients might well benefit from planned antibiotic therapy—that is, instituting, continuing, stopping, or altering antimicrobial treatment.

The hypothesis was proposed that there was a synergistic relationship between aerobic and anaerobic bacteria in the pathogenesis of surgical wound infection and the rest of the project was concerned with the development of an experimental model to prove it.

## Development of a bacterial counting method

Little interest has been shown by experimental pathologists in the quantification of mixtures of bacteria and consequently the only method available for counting the constituents of mixtures of *E. coli* and *B. fragilis* was a modification of the cumbersome Miles and Misra pour-plate technique *(15)*, which takes hours to perform and costs about £9 per result. The principle has been extended in a micromethod developed for counting aerobic organisms and is used in testing the bacterial purity of commercial beef and dairy produce *(16,17)*. I was able further to develop it for counting mixtures of *E. coli* + *B. fragilis (18,19)*, and full details and the experimental validation of the assay, the '4-plate technique', have been published *(18)*.

## Guinea-pig experimental wound model

The commonest aerobe found in the clinical trials was *E. coli* and the commonest anaerobe was *B. fragilis*. Representative standard strains of these organisms were therefore chosen from the National Collection of Type Cultures (NCTC), being *E. coli* NCTC 9434 and *B. fragilis* NCTC 9343. A single volume of 100 ml of culture was obtained for each organism and 0.5-ml portions stored frozen. The viability of the frozen bacteria remained constant and as a result, after thawing, any combination of organisms could be prepared with the confidence that subsequent testing would confirm the predicted values.

Guinea-pigs were chosen as the skin and subcutaneous fat, particularly in the shoulder region, seemed the most similar to those of the human abdominal wall (Gluksman, A. Personal communication, 1975). Under neuroleptanalgesia *(20)* four measured 2-cm wounds were incised with a scalpel on the freshly shaven, povidone–iodine-washed suprascapular area of each animal through skin and fat but not muscle. Each wound was inoculated with 0.1 ml of a bacterial suspension containing dilutions of either organism or their mixtures, freshly prepared as described above. The incisions were each closed with two sterile 12-mm Michel clips and the operation site was covered with an adherent vapour-permeable plastic dressing, Op-site *(21)*.

The guinea-pigs were inspected daily for liveliness. Wound induration was measured by palpation and rectal temperatures were taken.

On the seventh day the animals were killed and 4×2-cm blocks of tissue each containing one wound were excised down to, but not through, the deep fascia. Each tissue block was weighed, placed in 10 ml sterile saline, and homogenised in a Colworth stomacher *(22)*, and the viable bacterial content of the centrifuged supernatant obtained from the 'wound homogenate' was determined.

## Experimental design

The initial experiments were undertaken to establish the minimal infective and lethal doses of the organisms. The effect of using a mixture of *E. coli* + *B. fragilis* was then tested and, since pathogenic synergy was manifestly demonstrable, it was further evaluated by measuring the results of altering the components of the challenge inocula, keeping the amount of one organism constant while varying the concentration of the other.

## Results

Initial experiments confirmed the well-known finding that guinea-pigs are relatively susceptible to *E. coli* but totally impervious to *Bacteroides* even when inoculated with the neat culture. A subinfective challenge dose of *E. coli* ($9 \times 10^4$ per 0.1 ml) was identified. An initial synergy experiment used this subclinical dose of *E. coli* and a matched dose ($9.3 \times 10^4$ per 0.1 ml) of *B. fragilis*. Both these doses separately were subinfective, and a week later these wounds were histologically indistinguishable from a cleanly incised un-

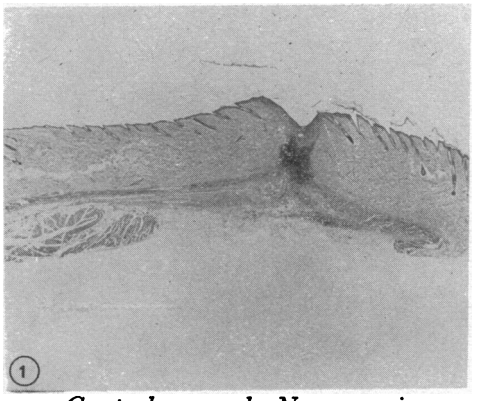

FIG. 1 *Control wound. No organisms were introduced at operation. The guinea-pig was killed 1 week later. This wound is healing by first intention. Haematoxylin and eosin (HE) × 10.*

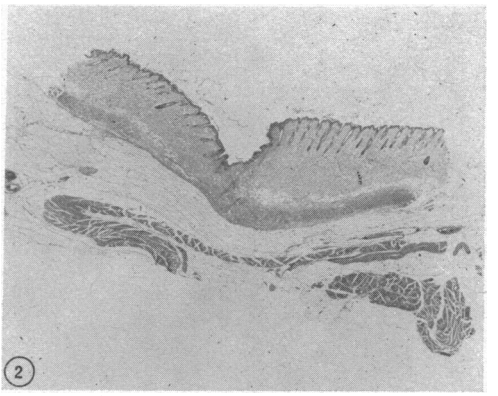

FIG. 2 *Wound contaminated with pure E. coli 9.0 × $10^4$ cells 1 week earlier. The wound is healing without inflammation. HE × 10.*

contaminated control wound (Figs 1–3). Nevertheless small numbers of bacteria ($10^5$ *E. coli* and $10^3$ *B. fragilis* respectively) were isolated from each of the wounds contaminated earlier with one or other of the organisms.

When 16 wounds were contaminated with a mixed inoculum containing the same *total* number of bacteria (9.2 × $10^4$ per wound), but half being one organism and half the other (4.5 × $10^4$ *E. coli* + 4.7 × $10^4$ *B. fragilis*), the guinea-pigs became listless, unwell, and pyrexial (38–40°C) by the second day. At one week, just before being killed, the animals were still listless and pyrexial and frank pus was just starting to point through the lines of the incisions. Massive induration of the wounds was obvious, ranging from 10 to 30 mm (mean 16, SEM 3.7) across the wounds. The homogenates yielded $10^7$–$10^8$ *E. coli* + $10^7$–$10^8$ *B. fragilis* per wound—that is, the two organisms were present in approximately equal numbers.

The microscopic appearances of these wounds (Fig. 4) show a striking difference from those of the previous wounds (Figs 1–3). In the mixed infection (Fig. 4) the changes associated with profound inflammation extend from the base to the surface of the wound, whose centre has been replaced by a mass of frank pus. Examination of the sections under

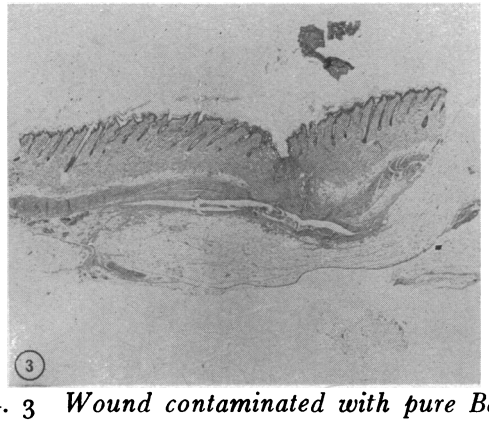

FIG. 3 *Wound contaminated with pure Bacteroides fragilis 9.3 × $10^4$ cells 1 week earlier. The wound is healing without inflammation. HE × 10.*

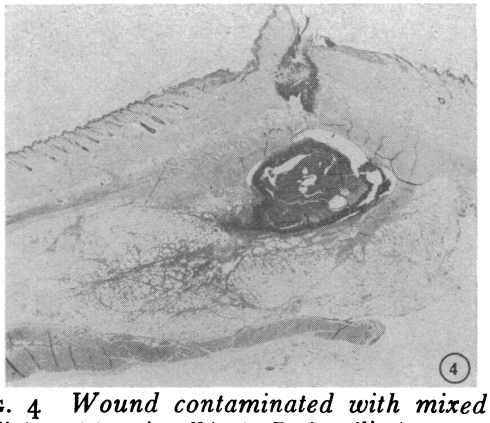

FIG. 4 *Wound contaminated with mixed E. coli (4.5 × $10^4$ cells) + B. fragilis (4.7 × $10^4$ cells) 1 week earlier. This wound shows severe inflammation with copious frank pus. HE × 10.*

higher magnification confirms these findings.

This series of experiments therefore shows unequivocally that, in the guinea-pig model, similar small infective doses of pure *E. coli* or pure *B. fragilis* failed to produce inflammation, whereas an inoculum of the same volume containing the same total number of organisms, half of which were *E. coli* and half *B. fragilis,* produced marked inflammation with the formation of frank pus. This is experimental pathogenic synergy in vivo and has been published *(23,24)*.

## Effect of E. coli : B. fragilis ratio on pus formation

The effects of altering the ratio of *E. coli* to *B. fragilis* in the challenge inoculum were examined as follows. Since it had been shown that $10^4$ *E. coli* + $10^4$ *B. fragilis* produced copious pus, the amount of one organism in the inoculum was held at $10^4$ while the concentration of the other varied. The yields of both organisms from the wound homogenates are shown in Figure 5. When the numbers of *B. fragilis* in the challenge were held at $4.7 \times 10^4$ while the concentration of *E. coli* was varied between $10^4$ and 0 the results summarised in Figure 6 were obtained (the dose range of *E. coli* in this section was limited because higher concentrations led to the guinea-pigs developing septicaemia). Each point on the two graphs represents the mean of at least 8 homogenised wounds, sometimes more. The standard error was approximately 0.5 log units for the higher values but 1.5 log units for the low ones.

Both graphs show that frank pus was formed only when the inocula contained at least $10^3$ *E. coli* and at least $10^4$ *B. fragilis*. Once pus had been formed the numbers of bacteria in the initial inocula appeared to have little effect on the numbers of bacteria subsequently obtained from the wound homogenates, which always lay between $10^7$ and $10^8$. Furthermore, when pus was present, provided that the numbers of *E. coli* and *B. fragilis* exceeded the threshold values of $10^3$ and $10^4$ cells respectively, their ratio in the inoculum did not seem to affect their ultimate concentration in the wound, which always contained approxi-

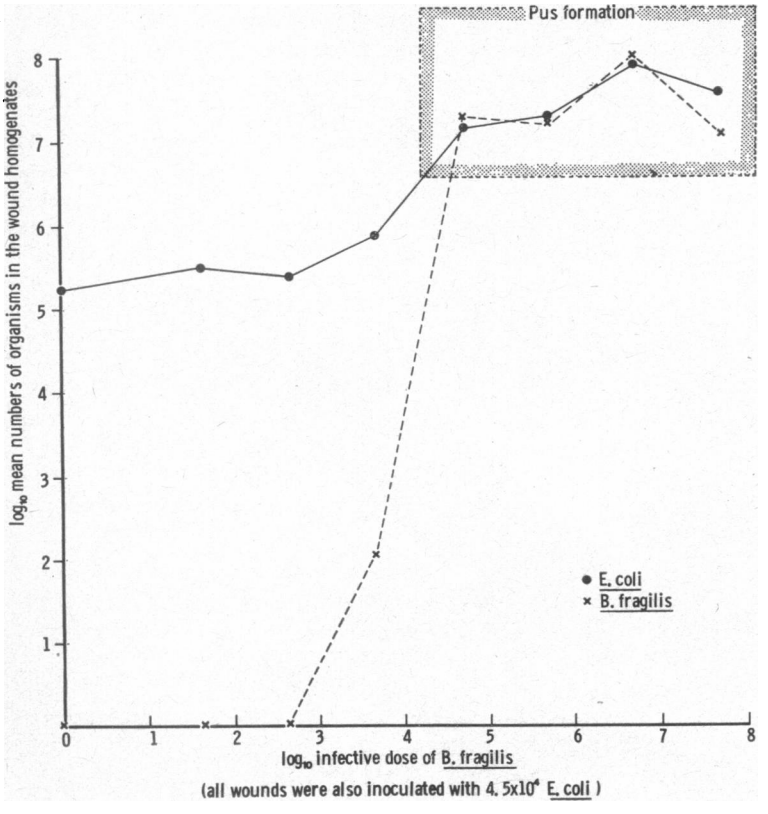


FIG. 5 *Effects of altering the E. coli:B. fragilis ratio in the challenge inocula on the later wound homogenate yields. Challenge inocula all contained E. coli* ($4.5 \times 10^4$ *cells*) *while the amount of B. fragilis varied from* $10^8$ *to* 0.

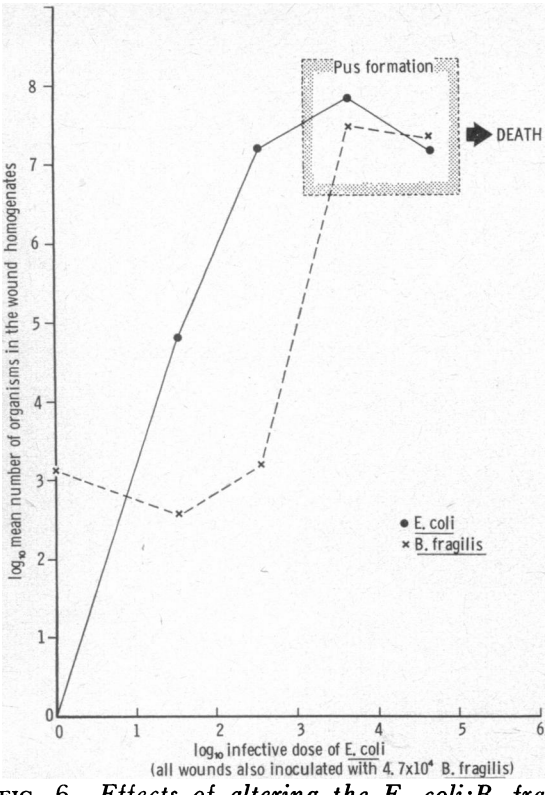


FIG. 6 *Effects of altering the E. coli:B. fragilis ratio in the challenge inocula on the later wound homogenate yields. Challenge inocula all contained B. fragilis (4.7 × 10⁴ cells) while the amount of E. coli varied from 10⁴ to 0. Higher doses of E. coli resulted in early fatal septicaemia (see text).*

mately equal numbers of aerobes and anaerobes. This is strong evidence for the presence of bacterial synergy.

As the dose of *B. fragilis* was decreased in mixed challenges the yield of this organism from the wound homogenates dropped rapidly to zero, while the yield of *E. coli* (held constant in the inoculum at $4.5 \times 10^4$) remained steady at $10^5$ cells. This was within the range of yields obtained when pure *E. coli* was inoculated alone. Thus at lower doses *B. fragilis* had no effect. A similar phenomenon is depicted for *B. fragilis* in Figure 6.

The results shown in Figues 5 and 6 confirm the earlier finding that relatively large numbers of pathogens could be cultured repeatedly from the homogenates of contaminated wounds that were apparently healing well by first intention.

## Discussion

Guinea-pigs are known to be fairly susceptible to pure *E. coli (25)*, as are jaundiced rabbits *(11)* but not rats *(26)*. The well-known finding that inoculation of an experimental animal with pure *B. fragilis* fails to cause any lesion *(8,9)* was confirmed in the present study and it was noted that pure cultures of the contaminating bacteria were later recovered from many of the wounds that had not formed pus and were apparently healing by first intenion. This observation has been made before and it has been suggested that in dirty human wounds the concentration of organisms present may form a good guide to whether it might be safe to proceed to delayed primary suture or not *(27)*.

The initial synergy experiment with $10^4$ *E. coli* + $10^4$ *B. fragilis* produced macroscopically visible pus and showed quantitatively and histologically that when small, subinfective inocula of the two organisms were mixed the combination caused gross inflammation with frank pus from which both organisms could be obtained in much higher concentrations ($10^7$–$10^8$).

This phenomenon of pathogen synergy was further evaluated in the experiments summarised in Figures 5 and 6. In each case, when the pus had been formed, the wounds contained large and strikingly similar quantities of organisms ($10^7$–$10^8$). For both organisms in turn, as the concentration of one bacterium was reduced a threshold was reached ($10^3$ *E. coli* or $10^4$ *B. fragilis*) below which pus was not produced and the wound concentrations of the organism that had been held constant in the original inoculum reverted to values ($10^5$ *E. coli* and $10^3$ *B. fragilis*) similar to those obtained after contamination with the pure culture and the concentration of the other organism fell towards zero.

The observation that in all the cases of overt infection with pus formation both *E. coli* and *B. fragilis* were present in the wound homogenates in large and equal numbers is further strong evidence for the existence of pathogenic synergy. The mechanism of such synergy has yet to be elucidated and may well depend on an interrelationship between relative oxygen tensions, soluble factors, and induced inhibition of leucocytic phagocytosis *(28)*. Such a mutual bacterial synergy has

been described in veterinary practice between the anaerobic *Fusiformis necrophorus* and the aerobic *Corynebacterium pyogenes* in sheep foot abscess *(29,30)*.

The guinea-pig model clearly possesses considerable potential for use in further basic science experiments to unravel the mechanisms of this synergy both in vivo and in vitro in terms of oxygen tension, soluble factors, and cell walls. Perhaps of more direct interest to surgeons and to their patients is the need to quantify and validate short- and long-term regimens of antibiotics, whether used preoperatively, peroperatively, postoperatively, or all three. Thus eventually we may hope to prescribe antibiotics for our bowel surgery which are specific for the organisms, and for the *combinations of organisms* involved, and this should lead to a reduction in morbidity and mortality.

## Conclusion

Evidence from surgical patients confirmed that there was a clinical *association* between wound infection and the isolation of mixed aerobic and anaerobic bacteria from operative wound swabs. This is all that such clinical studies are capable of doing—that is, demonstrating an association—because the scientific proof could only be produced by our being prepared to contaminate clean wounds in our patients with pathogenic bacteria, and this is plainly unethical. Thus we must turn to animal experiments.

A realistic quantified experimental animal model proved the existence of this synergy between *E. coli* and *B. fragilis* in guinea-pig wounds, fulfilling in a composite fashion Koch's third postulate and suggesting a theoretical explanation for the clinical success of metronidazole.

I am deeply indebted to my colleague and friend Dr R E Warren, Consultant Bacteriologist, Addenbrooke's Hospital, Cambridge, for hours of debate and guidance over the swab trials and for verifying all my culture plates. It is a pleasure to acknowledge the wholehearted co-operation of the surgical and nursing staffs at Addenbrooke's Hospital in providing the specimens I required, and I thank Dr B M Herbertson, Reader in Histopathology, University of Cambridge, for review of the slides.

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