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# Experimental infection of the bovine udder with *Campylobacter coli/jejuni*

By K. P. LANDER and K. P. W. GILL
*Central Veterinary Laboratory, New Haw, Weybridge, Surrey, U.K.*

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## SUMMARY

Five quarters of the udders of two lactating cows were infected by intramammary inoculation with *Campylobacter coli/jejuni* in doses ranging from 2·6 colony-forming units (c.f.u.) to $3{\cdot}8 \times 10^9$ c.f.u. The infected quarters developed clinical mastitis and the campylobacters were reisolated in large numbers from the milk. The milk from the uninfected quarters, and blood and faeces remained free of the organisms. The campylobacters could only be isolated by incubation of culture plates in a microaerobic atmosphere. The results showed that *C. coli/jejuni* can cause mastitis in the cow and that the bovine udder is a potential source of *C. coli/jejuni* in raw milk.

## INTRODUCTION

Several recent reports have associated outbreaks of *Campylobacter* infection in man with the consumption of bovine milk (Robinson *et al.* 1979; Taylor, Weinstein & Bryner, 1979; Public Health Laboratory Service, unpublished information). Although there is no direct proof of such an association, the circumstantial evidence is fairly strong, and in some cases has pointed to particular farms as the probable source of *Campylobacter* – contaminated milk. The way in which the organisms gain access to the milk has not been identified but Robinson *et al.* (1979) concluded that the milk was probably contaminated with bovine faeces. It seems unlikely that faecal contamination could account for the larger outbreaks of disease and more probable that, for these at least, a purer and more abundant source of campylobacters would be necessary. It seemed possible that the bovine udder could constitute such a source although there have been no previous reports of the excretion of *Campylobacter* sp. in milk. This paper reports on the ability of organisms of the *Campylobacter coli/jejuni* group (Veron & Chatelain, 1973; Duffell & Skirrow, 1978) to establish infection and multiply within the bovine udder.

## MATERIALS AND METHODS

### *Bacterial strains*

For the first experiment the *Campylobacter* strain used was supplied by Dr A. T. Willis of Luton Public Health Laboratory and was designated Strain 12676. It had been isolated from the faeces of a child who was one of approximately 2500 children affected in an outbreak of diarrhoea and vomiting associated with *C. coli/jejuni*.

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This was thought to be related to the consumption of free school milk supplied by a single retailer (Willis, A. T., personal communication). It is not known precisely how many times the organism had been subcultured before its arrival at this laboratory. On receipt it was identified biochemically as *Campylobacter coli/jejuni*. It was subcultured once, suspended in M/15 phosphate buffered saline, pH 7·0, containing 15% dimethyl sulphoxide, and stored in liquid nitrogen at a temperature of −196 °C until required.

For the second experiment the strain used was isolated from one of the infected quarters of the cow inoculated in the first experiment. It was designated Strain V212X. It was biochemically identical with Strain 12676. After isolation, it was cloned once and then stored in liquid nitrogen as described above until required.

*Culture methods*

The media used for routine plating were 10% sheep blood agar (BA) and a modification of the selective medium of Skirrow (1977) comprising 7% lysed horse blood agar containing vancomycin (10 μg/ml), trimethoprim (5 μg/ml), polymyxin B (2·5 i.u./ml) and actidione (100 μg/ml) (SKA).

Broth media included veal infusion broth (Difco Laboratories) (VIB) and a selective and enrichment medium comprising VIB plus 7% lysed horse blood, 1% bacteriological charcoal (Oxoid Ltd.), vancomycin (40 μg/ml), trimethoprim (20 μg/ml), polymyxin B (10 i.u./ml), actidione (100 μg/ml) and 5-fluorouracil (500 μg/ml) (TM) (K. P. Lander, to be published).

The broth media were dispensed in volumes of approximately 20 ml in screw-topped Universal bottles.

For the culture of milk samples, volumes of approximately 0·05 ml of milk were spread over the surfaces of BA and SKA plates for aerobic and microaerobic incubation. After incubation the plates were examined and an estimate was made of the number of colonies of *C. coli/jejuni* on the plates. In this way the excretion rate of campylobacters in the milk could be roughly measured.

Faeces samples were cultured on BA and SKA and enriched in TM broth.

Blood samples in 5 ml volumes were inoculated into 20 ml VIB immediately after collection.

Plates were routinely incubated microaerobically at 37 °C for 2 days in a gassed incubator (Heraeus Type B 5060 EK/$O_2$) adjusted to contain an atmosphere of 6% oxygen, 10% carbon dioxide and 84% nitrogen with 75% relative humidity. For milk samples replicate plates were also incubated at 37 °C for 2 days in air, and on 2 occasions further replicate plates were incubated at 37 °C for 2 days in 10% carbon dioxide in air.

The broth media were incubated at 37 °C for 2 days in air and then subcultured onto BA and SKA plates for microaerobic incubation.

*Preparation of inoculum*

A vial of the required bacterial strain was taken from liquid nitrogen storage and thawed at room temperature. The contents were spread over 4 BA plates and incubated microaerobically for 2 days at 37 °C. After checking that the cultures

were pure and contained less than 10 % of coccoid forms of *Campylobacter* the cells were suspended in $\frac{1}{4}$ strength Ringer's solution.

For the first experiment 20 ml of a suspension of Strain 12676 was diluted to an opacity corresponding to MacFarland tube 2, which was estimated to contain approximately $10^7$ organisms per ml. Volumes of 5 ml of this suspension were used as the inoculum and inoculation was carried out within 30 min of preparing the suspension.

For counting the viable organisms in the suspension tenfold dilutions of the suspension were made in 10 ml volumes of $\frac{1}{4}$ strength Ringer's solution using a fresh pipette for each dilution. Volumes of 0·1 ml of concentrations $10^{-3}$ to $10^{-7}$ were spread over each of 2 BA and 2 SKA plates and incubated microaerobically at 37 °C for 2 days. The colonies from concentrations $10^{-6}$ and $10^{-7}$ were counted and the number of living organisms was calculated by a weighted mean.

For the second experiment, Strain V212X was used and the suspensions, dilutions and colony counts were performed as for the first experiment. For this experiment 4 different inocula were prepared containing graded doses of bacteria.

*Animals*

Two Guernsey cows in mid-lactation were used, one for each experiment. The cows were examined before inoculation and found to be clinically healthy. Pre-inoculation bacterial cultures of milk, faeces and vaginal mucus were negative for *Campylobacter* sp.

Cow 1 for the first experiment was aged $5\frac{1}{2}$ years and Cow 2 for the second experiment was aged $8\frac{1}{2}$ years.

The animals were housed in separate loose boxes, fed normally and milked twice daily with separate milking machines. In the first experiment a normal bucket unit was used. In the second experiment an individual quarter milking unit was used to prevent the transfer of infection between quarters during milking. After each milking the teats were dipped in a solution of sodium hypochlorite containing 1 % available chlorine.

*Animal inoculation*

The inoculum of 5 ml of suspension was drawn into a 5 ml plastic syringe. To this was attached a 40 mm 11/10 (19G × $1\frac{1}{2}$ in) hypodermic needle the sharp end of which was covered with a 20 mm length of surgical quality boilable polythene tubing (1·7 mm external diameter, 1·0 mm bore). The ends of the polythene tubing were filed smooth with a nail-file. The needle and attached tubing were boiled for 15 min before use. A separate syringe, needle and tubing was used for each inoculation.

The inoculations were carried out after afternoon milking. The end of the teat was rubbed vigorously with a cotton-wool swab soaked in 70 % ethanol. Then the polythene tubing attached to the needle and syringe was passed through the teat canal to a depth of approximately 15 mm and the inoculum instilled into the teat sinus. The inoculum was gently massaged up into the teat cistern and then into the quarter.

In the first experiment the right fore (RF) and left hind (LH) quarters of Cow 1 were inoculated with suspensions containing $3{\cdot}8 \times 10^9$ colony-forming units (c.f.u.) of *C. coli/jejuni* Strain 12676. The left fore (LF) and right hind (RH) quarters were inoculated with 5 ml of sterile $\frac{1}{4}$ strength Ringer's solution.

In the second experiment the four quarters of Cow 2 were inoculated with graded doses of *C. coli/jejuni* Strain V212X. The LH quarter received $2{\cdot}6 \times 10^2$ c.f.u., the LF $2{\cdot}6 \times 10^1$, the RH $2{\cdot}6 \times 10^0$ and the RF $2{\cdot}6 \times 10^{-1}$ c.f.u.

*Sampling procedures*

Milk samples were taken daily before morning milking using the method recommended by Griffin *et al.* (1977). Samples were cultured within one hour of collection. After culturing, the samples were processed for cell counting.

Faeces samples were collected fresh from the rectum. They were cultured daily for at least 3 days before and 10 days after infection.

Blood samples were taken by syringe and needle from the jugular vein. Samples were collected daily for 3 days during the height of the febrile reaction.

*Cell counts*

Cell counts on milk samples were done by electronic particle counter (Coulter Electronic Co. Ltd) according to the method recommended by the International Dairy Federation (Anon, 1971).

*Clinical examination*

Clinical examination was carried out daily and included taking the rectal temperature, palpation of the udder and observation of general demeanour, appetite, respiratory rate and consistency of faeces. In addition the appearance of the milk was observed both in a strip-cup and in the sampling tube.

## RESULTS

The results of the observations on bacterial isolation from milk, cell counts and rectal temperatures are summarized in Fig. 1.

In the first experiment where a large inoculum was used *C. coli/jejuni* was recovered from the infected quarters at the first milking after inoculation and thereafter for a further 5 days. The peak rate of excretion of bacteria was reached on the second day. No campylobacters were recovered from the quarters which were inoculated with the suspending fluid only.

In the second experiment, with much smaller inocula, the organisms apparently disappeared from the milk in the LF and LH quarters for the first 24 hours and from that in the RH quarter for the first 36 hours. Then there was a gradual increase in the numbers of bacteria recovered from these 3 quarters until all reached a maximum rate of excretion on day 5. The RF quarter which, according to the viable count on the inoculum, probably received no bacteria shed no *Campylobacter* and showed no evidence of the mastitis seen in the other 3 quarters.

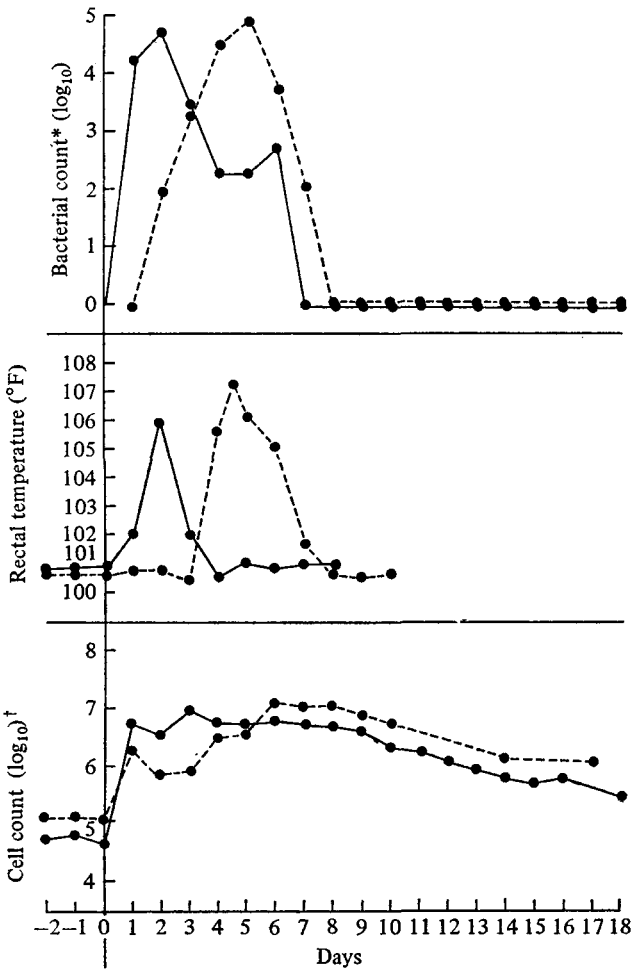


Fig. 1. Cell counts and approximate bacterial counts in milk, and rectal temperatures of two cows with mastitis caused by *Campylobacter coli/jejuni*. * Mean of infected quarters (bacteria/ml. foremilk. † Mean of infected quarters (cells/ml. foremilk). Cow 1 ●——●, Cow 2 ● - - - ●.

In both animals campylobacters were recovered in large numbers from the milk for only about 3 days after which the infection was eliminated fairly rapidly.

During the course of the experiment no organisms other than *C. coli/jejuni* were isolated in significant numbers from the milk.

The cell counts in all quarters showed an initial rise following inoculation. In the uninfected quarters these reached a mean of $1{\cdot}3 \times 10^6$ cells/ml on the day after inoculation and by day 4 had returned to counts of less than $2{\cdot}5 \times 10^5$/ml. The infected quarters showed a second, greater rise reaching a peak in both cows the day after the highest bacterial counts. The cell counts in all quarters returned to preinoculation levels between 5 and 6 weeks after inoculation.

The rectal temperatures became greatly elevated during the course of the infection, reaching 107·3 °F (41·8 °C) in Cow 2. The highest temperatures coincided with highest rates of excretion of bacteria. The febrile reaction was accompanied

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by listlessness, depression, shivering, inappetance and mild diarrhoea. The infected quarters became indurated, swollen, hot and tender and the RH quarter of Cow 2 became oedematous. The induration and swelling developed overnight coinciding with the highest temperature and peak rate of bacterial excretion. It remained unchanged for about 2 days and then slowly resolved during the next 5 to 7 days. By the results shown in Fig. 1 and by subjective assessment Cow 2 was more severely affected than Cow 1 in spite of receiving smaller inocula.

The visual appearance of the milk was altered in the affected quarters. In the early stages as the bacterial excretion rate was reaching a peak the milk was only slightly watery and had a granular deposit. This was hardly noticeable in the strip-cup but was more obvious in the sampling tube. About 2 days later when the bacterial counts in the milk had dropped considerably the secretion became very watery and yellow or straw-coloured with numerous stringy clots. The milk slowly returned to normal appearance over the next 4–5 days.

The milk yield was severely depressed during the febrile reaction and then slowly improved. However neither cow returned to her normal milk yield and at 6 weeks postinoculation both cows were giving about one half of their preinoculation yields.

Cultures of faeces and blood were negative for *C. coli/jejuni* throughout the experiment.

In six milk samples where replicate plates were incubated microaerobically, in air and in 10% carbon dioxide in air, only the microaerobic plates showed large numbers of *C. coli/jejuni*; the other two sets of plates showed no bacterial growth.

## DISCUSSION

The results of these experiments show that *Campylobacter coli/jejuni* can establish infection in the bovine udder even from very small inocula, that it can produce clinical mastitis in the cow and that infection results in the shedding of large numbers of campylobacters in the milk.

If it is accepted that *C. coli/jejuni* is a primary cause of enteritis in man then milk is potentially an important vector of yet another human disease. The first suggestion that milk-borne transmission to man might have occurred was made by Levy (1946). In the last 2 years circumstantial evidence for this mode of transmission has been accumulating (Robinson *et al.* 1979, Taylor *et al.* 1979, Public Health Laboratory Service, unpublished reports).

The question of how milk becomes contaminated with campylobacters has not yet been answered. Robinson *et al.* (1979) concluded that, in the absence of any evidence suggesting that campylobacters were excreted in milk, the outbreaks they described probably resulted from faecal contamination of milk. It is known that *C. coli/jejuni* can be isolated from the faeces of a proportion of cattle (Elazhary, 1968) and that it may be the cause of diarrhoea in adult and young cattle (Jones, Orcutt & Little, 1931). It is not known in what numbers the campylobacters are excreted in bovine faeces, nor is there any published information on the infective dose of *C. coli/jejuni* for man. Nevertheless it seems to us reasonable to assume that bovine faeces would be unlikely to provide a sufficiently abundant

inoculum in milk to infect large numbers of people without the faecal contamination becoming obvious either macroscopically, by spoiling or souring of the milk, or through the routine tests for cleanliness of milk. The nature of the initial source of contamination is of particular significance with *Campylobacter* spp. because it is unlikely that there would be extensive multiplication of these organisms in the milk after it has left the cow. This is chiefly because very few human strains of thermophilic *Campylobacter* spp., and none of the British strains isolated from suspected milk-borne outbreaks of disease, are able to multiply at temperatures below 30·5 °C (M. B. Skirrow, personal communication). Even at adequate temperatures they multiply comparatively slowly and would probably be outgrown by many of the souring and spoiling bacteria normally present in raw milk, and present in much greater numbers in milk contaminated with faeces.

These experiments show that the infected bovine udder could provide a pure and abundant source of *C. coli/jejuni* in milk. Furthermore the sequence of events following experimental inoculation indicates that the udder could excrete quite large numbers of organisms before there were any obvious signs of disease in the cow. Even at the time of greatest excretion of bacteria the milk itself was not grossly abnormal. It can be postulated that, if milder cases occur, the sudden onset and fairly rapid resolution of the mastitis could go unnoticed altogether, particularly with modern highly mechanized and streamlined milking routines.

As far as is known this is the first published report of bovine mastitis caused by *Campylobacter* sp. The clinical picture resembled in many respects the mastitis caused by coliform organisms (Marr, Jackson & Robinson, 1979), including the sudden onset, the fairly severe illness, the marked local reaction in the affected quarters and the nature of the secretion in both the early and later stages of the disease. The similarity may extend to the method of infection since the size of the lowest infective dose (2·6 colony-forming units) suggested that under natural conditions infection could easily result from faecal contamination of the teat orifice. It is possible that *C. coli/jejuni* will prove to be one of the previously undiagnosed causes of bovine mastitis. If it is to be recognized, our results suggest that cultures from suspected milk samples will have to be incubated in microaerobic conditions.

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