THE FIRST ISOLATIONS OF *CAMPYLOBACTER MUCOSALIS* FROM PIGS IN SOUTH AFRICA

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ABSTRACT

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The first isolations of *Campylobacter mucosalis* in South Africa are described. Isolations were made from a 6-week-old weaner pig with necrotic enteritis and from 2 gingival swabs of suckling piglets from herds with histories of porcine intestinal adenomatosis. The isolates were serologically identified as being serotype A strains.

INTRODUCTION

Campylobacter mucosalis has been isolated world-wide from clinical cases of the disease complex known as porcine intestinal adenomatosis (PIA) (Rowland & Lawson, 1981). Although clinical symptoms of the disease were reported as early as 1931 (Biester & Schwarte, 1931), it was only in 1974 that a previously unknown organism was isolated from clinical cases (Lawson & Rowland, 1974). This organism was named *C. sputorum mucosalis* by Lawson & Rowland, 1974 but later renamed as *C. mucosalis* (Roop, Smibert, Johnson & Krieg, 1985a). A 2nd, previously unknown organism, *C. hyointestinalis,* was isolated in 1983 from PIA cases (Gebhart, Ward, Chang & Kurtz, 1983). *C. mucosalis* can be isolated only from clinical cases of PIA and the oral cavity of some healthy pigs in herds in which PIA occurs (Roop *et al.*, 1985b). *C. hyointestinalis,* on the other hand, can be isolated from pigs with PIA or other enteric disease, as well as from healthy cattle and cattle with diarrhoea (Morgan & Bland, 1985). However, conclusive evidence for either or both of these organisms being the sole or primary aetiological agents of PIA does not exist (Boosinger, Thacker & Armstrong, 1985), as the disease has not been successfully reproduced by the mere inoculation of either one or both of these organisms into pigs.

In South Africa, clinical signs of the disease complex on a herd basis were observed as early as 1957 (Loveday, personal observations, 1957). Intracellular curved bacteria have been seen in Warthin–Starry stained histology sections of cases with PIA submitted to the Veterinary Research Institute (VRI), Onderstepoort, since 1980 (Williams; Van der Lugt, personal communications, 1986). However, the culture of either *C. hyointestinalis* or *C. mucosalis* until recently has been unsuccessful. The first isolation of *C. hyointestinalis* in South Africa has been recently documented by Van der Walt & Van der Lugt (1988).

The first isolations of *C. mucosalis* strains from the oral cavity of piglets and from an animal with the necrotic enteritis form of PIA are described here.

MATERIALS AND METHODS

History of the necrotic enteritis case

A 6-week-old weaner pig from a large commercial piggery near Middelburg, Transvaal, died with signs of severe wasting. A routine post mortem examination revealed that the ileum showed signs of necrotic enteritis (NE) superimposed on a thickened mucosa. Previous necropsy evidence of the PIA complex on this piggery had been identified as well as regional ileitis (RI) and proliferative haemorrhagic enteropathy (PHE) in finisher pigs. No antibacterial drugs were added to any of the feeds used by the piggery since the losses from the PIA complex had been too low to justify the cost for feed medication.

Isolation procedures

A portion of the terminal ileum showing necrotic enteritis was bound off, chilled (4 °C) and submitted the following day to the laboratory. The method for the isolation of *C. mucosalis* was as described by Lawson & Rowland (1974). The piece of ileum was cut open and rinsed with phosphate buffered saline (pH 7,2). The mucosa was scraped with a scalpel blade and a 1 g aliquot was suspended in reinforced clostridial medium. This was blended at maximum speed for 30 s in an Ultra-turrax[1] homogenizer.

The volume of the medium was made up to 20 mℓ (1/20 dilution). Subsequently, 1/40 and 1/80 dilutions of the medium were made, and 0,1 mℓ amounts were seeded onto Columbia agar plates (CBA) and CBA plates containing the following antimicrobials: novobiocin[2] (5 μg/mℓ), trimethoprim[1] (5 μg/mℓ) and brilliant green[3] (1/80 000) (Lawson & Rowland, 1984).

The plates were incubated for up to 5 days at 37 °C in anaerobic jars[4] which contained an H_2-microaerobic atmosphere, obtained by the evacuation of the jars to −560 mm Hg and replacing the air with 15 % CO_2 in 85 % H_2. When this gas-mixture was unavailable, an alternate mixture was used. Anaerobic jars were evacuated to −380 mm Hg and filled with a mixture of 79 % N_2, 5 % H_2 and 16 % CO_2.

Gingival swabs

Two sets of swabs from 6 animals each were taken from 12 piglets from 2 different commercial piggeries, both of which had a history of sporadic cases of PIA. The swabs were taken by vigorously swabbing the gingival margin (Lawson, Rowland & Roberts, 1975) and transferring to a transport medium (Lawson, personal communication, 1986). The medium consisted of nutrient broth 13 g, proteose peptone 15 g, agar 3,5 g, sodium formate 2,5 g and 100 mℓ distilled water. The

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medium was sterilized and cooled to 56 °C and brilliant green 1/10 000, rifampicin 5 μg/mℓ and 5 mℓ of lysed horse blood were added aseptically. Small, screw-topped bottles were filled to the neck with the medium, the tops tightly closed and refrigerated until needed.

The swabs were broken off in the medium, the tops of the bottles tightly closed and sent to the laboratory in an ice-container. The swabs were removed from the transport medium, transferred to 5 mℓ of saline and vigorously shaken for at least 5 min. The saline was filtered through a 1,2 μm filter and the last drops plated out on CBA plates and on CBA plates containing antimicrobials. The plates were incubated in the H_2-microaerobic atmosphere as described.

Characterization tests

The tests described by Roop *et al.*, (1985b), were used to characterize the isolates. Growth at 25 °C, 42 °C and 45 °C was examined on CBA plates and H_2S production on triple sugar iron (TSI) agar slopes. Sensitivity to 0,001 % cephalothin[5], 0,003 % nalidixic acid[6] and 0,4 % tetrazoliumsalt[7] were examined on tryptose agar[8] plates containing 10 % horse blood[9]. Growth in the presence of 1,5 % NaCl was examined on agar plates (Lawson & Rowland, 1984), and growth in the presence of 1 % glycine, 3,5 % NaCl or 1 % oxgall was examined in *Brucella* broths[4] containing 0,16 % agar. A *Brucella* broth with no additive was also inoculated as a control. Nitrate reduction was examined in a liquid broth (Mac-Faddin, 1980). All tests were read after 48–72 h incubation in either the H_2-microaerobic atmosphere or the alternate atmosphere. *C. mucosalis* strain NCTC 11 000 was included in all the tests as a reference.

Antigenic examinations

All 3 isolates were examined serologically by means of a microtitre agglutination test to determine their serotype.

Production of antisera

Antisera were produced against all 3 serotypes, A, B and C, of *C. mucosalis*, the cultures being obtained from the National Collection of Type Cultures[11]. The method followed was as described by Chang, Kurtz, Ward & Gebhart, 1984. Growth from agar plates was suspended in PBS containing 0,4 % formalin. Cells were washed and adjusted to an optical density of 0,35 absorbance at 600 nm with a Bausch & Lomb Spectronic 21 spectrophotometer[10]. Rabbits were injected intravenously with 1, 2, 2, 4 and 4 mℓ quatities over a 2-week period and bled 10 days after the last injection. Serum was separated and stored in 2 mℓ aliquots at -18 °C until used.

Antigen for the agglutination test

Antigen was produced as described by Lawson, Rowland & Roberts, 1976. Growth from the 48 h plates was suspended in PBS containing 1 % formalin, left overnight at 37 °C, and washed. The density was nephelometrically[12] adjusted to a reading of 72 on the Eel scale, which was equivalent to Brown's opacity tube 2. Antigens were kept refrigerated until used.

[5] Keflin, Lilly Laboratories (S.A.) (Pty) Ltd, Short Street, Isando 1600
[6] Labretoria (Pty) Ltd, P.O. Box 20295, Alkantrant, Pretoria 0005
[7] BDH, Merck (S.A.) (Pty) Ltd, P.O. Box 3497, Johannesburg 2000
[8] Difco, Laboratory & Scientific Equipment Co. (Pty) Ltd, P.O. Box 45125, Mayfair, Johannesburg 2108
[9] Section of Reproduction, Veterinary Research Institute, working document
[10] Protea Laboratories, P.O. Box 39127, Bramley, Johannesburg 2018
[11] Colindale, England
[12] Vickers Instruments, P.O. Box 6378, Dunswart, Johannesburg 2211

Microtitre serum agglutination test

A previously described method (Van der Walt, 1987) was adapted. Serial doubling dilutions of sera with PBS leaving 50 $\mu\ell$ aliquots ranging from 1:40 to 1:10 240 were carried out in microtitre plates[13], with a Micro Compu-Pet Multi Diluter[14]. Antigen (50$\mu\ell$) was added to each well, and the microtitre plates were incubated for 90 min at 37 °C on a shaker. The plates were centrifuged for 1 min at 800 g to precipitate the agglutinate.

The agglutination reaction was read against a dark background with illumination from the sides. When agglutination occurred, cells were seen in either a granular clump or a granular shield around the bottom of the wells, always with a clear supernatant fluid. In the negative reaction the cells formed a button in the wells and the supernatant fluid was cloudy.

RESULTS

Ileum sample

The gross pathology of the ileum was consistent with the description of the NE form of PIA (Rowland & Lawson, 1981). A yellow cheesy mass covered the mucosa, while the gut wall of affected areas was thickened.

After 48 h of incubation small round translucent catalase-negative colonies were found on the plates containing antimicrobials of the 1/80 dilutions. Mediums which did not contain antimicrobials were too overgrown for the detection of the small colonies. The colonies had a dirty yellow colour when streaked onto white paper. Gram-stained smears revealed that the organisms from these colonies showed *Campylobacter* morphology, the cells being short, curved bacilli, while in older cultures coccoid bodies appeared. No catalase-positive colonies resembling *Campylobacter* were seen. The organism isolated from the ileum was designated C/44.

Gingival swabs

On the CBA-plates and plates containing antimicrobials made of 1 swab of each of the 2 groups of swabs, small, round, translucent colonies appeared after the 48 h incubation. These 2 isolates, C/21–1 and C/27–6, fitted the description of isolate C/44.

Characterization tests

The results of the characterization tests carried out on isolates C/44, C/21–1 and C/27–6, as well as the reactions of the reference strain *C. mucosalis* (NCTC 11000), are set out in Table 1. All 3 isolates, C/21–1, C/27–6 and C/44, were found to be *C. mucosalis*. Small differences in the reactions of the 4 strains examined and the description of *C. mucosalis* (Roop *et al.*, 1985a; 1985b) were seen. All 4 strains were resistant to cephalothin, whereas other workers found *C. mucosalis* to be sensitive to this substance (Table 1) (Roop *et al.*, 1985a; 1985b). Strain NCTC 11000 did not grow at 45 °C, while the 3 isolates were all able to grow to some degree at this temperature.

The use of either the H_2-atmosphere or the alternate microaerobic gas mixture did not markedly influence the reactions of the isolates, except that isolates C/21–1 and C/27–6 did not grow at 25 °C under the latter atmosphere. When this test was repeated in the H_2-atmosphere, these 2 isolates both grew at 25 °C.

[13] Linbro U-bottom microtitre plates, Seravac, P.O. Box 5933, Johannesburg 2000
[14] General Diagnostics, Warner Pharmaceuticals (Pty) Ltd, P.O. Box 1718, Cape Town 8000

TABLE 1 Characteristics of *Campylobacter* isolates, *C. mucosalis* and *C. sputorum*[1]

Characteristic	*Campylobacter* isolates			*C. mucosalis* strain NCTC[2] 11000		*C. sputorum*	
	C/44	C/21–1	C/27–6	*	†	biovar *sputorum*	biovar *bubulus*
Growth at:							
25 °C	+[3]	+	+	+	+	−[4]	−
42 °C	+	+	+	+	+	+	−
45 °C	+	(+)	(+)[7]	−	−	NK	−
H₂S production TS1	+	+	+	+	+	+/−[5]	+
Sensitivity to:							
cephalothin	−	−	−	−	+	+	+
nalidixic acid	+	−	−	+	+	−	−
tetrazolium	+	+	+	+	+	NK[6]	NK
Nitrate reduction	+	+	+	+	+	+	+
Growth in:							
1 % glycine	+	+	+	+	+	+	+
1,5 % NaC1 plates	−	−	−	−	−	+/−	+
3,5 % NaC1	−	−	−	−	−	−	+
1 % oxgall	+	+	+	+	+	+	+
control	+	+	+	+	+	+	+

[1] Roop *et al.*, 1985b
[2] Reference strain
[3] + = growth
[4] − = no growth
[5] +/− = variable
[6] NK = not known
[7] (+) = weak positive
* Results found in this study
† Results found by Roop *et al.*, (1985b)

TABLE 2 Agglutination reactions of the 4 *Campylobacter* strains with antisera produced from 3 serotypes of *C. mucosalis*

Strains used as antigen	Reciprocal agglutination titre of *C. mucosalis* antisera		
	Serotype A antisera	Serotype B antisera	Serotype C antisera
C. mucosalis serotype A[1]	320	—	—
C/44	320	—	—
C/21–1	160	ND[2]	—
C/27–6	10 240	ND	—

[1] Reference strain NCTC 11000
[2] ND = Not done

Serotype determinations

The agglutination reactions of strains C/44, C/21–1, C/27–6 and of NCTC 11000 with the 3 *C. mucosalis* antisera are set out in Table 2. Strain NCTC 11000, a serotype A, reacted to a high titre with the serotype A antisera but not with the serotype B or C antisera. All 3 local isolates reacted with serotype A antiserum (Table 2).

DISCUSSION

All 3 isolates, C/44, C/21–1 and C/27–6, conformed to the description of *C. mucosalis* (Roop *et al.*, 1985b) and were biochemically classified as *C. mucosalis* strains. As strain C/44 was isolated from a clinical case of PIA, there was little doubt over the isolate's classification. No other catalase-negative, *Campylobacter*-like organisms have been isolated from PIA-gut samples by other workers.

Strains C/21–1 and C/27–6 were isolated from the oral swabs of suckling piglets. The anaerobic to H₂-micro-aerobic flora of the pig's gingival crevice seem to consist of a varied population of curved organisms resembling *Campylobacter* (Lawson *et al.*, 1976). These campylo-bacter-like organisms can be isolated from the oral cavity of pigs from herds with a history of PIA (Lawson *et al.*, 1975). Some of these organisms were biochemically different from *C. mucosalis*, while others were biochemically similar. Of these latter organisms, some were antigenically closely related to *C. mucosalis*, whilst others were distinct (Lawson *et al.*, 1976; Lawson, Leaver, Pettigrew & Rowland, 1981). The agglutination of isolates C/21–1 and C/27–6 with serotype A antisera is an additional way of identifying these 2 isolates as *C. mucosalis*.

Isolate C/44 was identified as a serotype A strain of *C. mucosalis*. Serotype A is the serotype of *C. mucosalis* most commonly identified, while serotype B is scarce (Lawson *et al.*, 1981; Lawson *et al.*, 1976). It is not known whether serotype C is involved in clinical PHE (Lawson *et al.*, 1981). The identification of all 3 isolates as serotype A is in agreement with the findings of these workers that serotype A is the most often encountered serovar of *C. mucosalis*.

C. mucosalis is the only catalase-negative *Campylobacter* able to grow at 25 °C (Roop *et al.*, 1985b). However, various workers have found that some strains do not grow at 25 °C (Lawson & Rowland, 1984; Gebhart *et al.*, 1983; Ohya, Kubo & Watase, 1985). Ohya *et al.* (1985) found in their study that non-serotype A strains had a narrow growth temperature range around 37 °C. In addition to possible strain differences, fluctuations in the incubator temperature or the effect of gaseous environment, may explain these discrepancies.

The observation that the 4 *C. mucosalis* strains examined in this study were resistant to cephalothin

(Table 1) might have resulted from the use of 0,001 % cephalothin in the agar medium. The other workers (Roop *et al.,* 1985a), who found *C. mucosalis* to be sensitive to cephalothin, used discs containing 30 μg cephalothin. In this study, the *C. mucosalis* reference strain did not grow at 45 °C. This is in agreement with the findings of Lawson & Rowland (1984). However, the 3 local *C. mucosalis* isolates did grow at this temperature (Table 1). This characteristic may be a strain-specific characteristic of *C. mucosalis* strains.

Clinical signs of the PIA-complex were observed as early as 1957 in the Republic of South Africa. During the last 10 years, clinical cases have often been seen (Spencer, Loveday, personal observations 1976–1986). Outbreaks were sporadic and mostly affected single animals in herds. Signs of the disease are not restricted to a certain part of the country but occur endemic. The detection of *C. mucosalis* in an animal or in a herd remains difficult. *C. mucosalis* could not be shown in the gut of healthy animals (Chang *et al.,* 1984) but can be isolated from the oral cavity of healthy pigs from herds where PIA had occurred previously (Lawson *et al.,* 1975). PIA in a herd appears to be transient and many affected animals recover fully within 6 weeks (Rowland & Lawson, 1981). Some animals produce circulating antibodies against *C. mucosalis* and *C. hyointestinalis* following infection (Lawson & Rowland, 1984; Wilson, Chang, Gebhart, Kurtz, Drake & Lintner, 1986), but this response does not indicate if a specific animal would develope PIA, nor does it indicate immunity of an animal. However, the presence of a positive agglutination titre in animals in a herd or the positive isolation of *C. mucosalis* from the oral cavity of some animals in a herd indicate that there had been contact with *C. mucosalis,* but would not be an indication of the possibility of clinical disease. The only way to determine whether a specific animal has *C. mucosalis* in its intestine is either culturing of the organism from affected tissue or by the immunofluorescent staining of organisms in sections of affected tissues (Chang *et al.,* 1984).

Lawson *et al.* (1975) made isolations of *C. mucosalis* from the oral cavities of pigs from herds where PIA had occurred previously. The 2 oral isolations made in this study were also made from herds with previous histories of PIA, although clinical cases had been absent for some time. These isolations show the although clinical disease may be absent in a herd, the organisms may still be present. The presence of *C. mucosalis* in the oral cavity of pigs may be important in the epidemiology of PIA, but the way in which the organisms reach and invade the gut epithelium and cause disease remains unknown.

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