STUDIES ON *CAMPYLOBACTER SPUTORUM* SUBSPECIES *MUCOSALIS*

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PLATE XII

IN a previous communication, we have described the principal characters of a number of strains of *Campylobacter sputorum* originating from the intestinal mucosa of cases of porcine intestinal adenomatosis (Lawson, Rowland and Wooding, 1975*a*). The number of organisms examined in our initial work was necessarily restricted.

In an examination of 10 normal pigs, it was not possible to recover *C. sputorum* from the intestinal sites at which adenomatosis commonly occurs or from the intestinal contents (Lawson and Rowland, 1974).

Porcine intestinal adenomatosis (PIA), necrotic enteritis (NE), regional ileitis (RI) and proliferative haemorrhagic enteropathy (PHE) all have a common morphological basis; organisms that could not be differentiated from *C. sputorum* subspecies *mucosalis* have been isolated from pigs with the first three of these conditions (Rowland and Lawson, 1975). The present communication compares, in detail, *mucosalis* strains from adenomatous tissue with strains isolated from pigs with NE or RI and also from the oral cavity of pigs (Lawson, Rowland and Roberts, 1975*b*). To date, we have been unable to obtain fresh material for bacteriological examination from cases of PHE.

MATERIALS AND METHODS

Isolation procedures. These were the same as described previously (Lawson and Rowland, 1974). Dilutions of homogenised mucosa were prepared in Reinforced Clostridial Medium (RCM; Oxoid Ltd) and dilutions of chyme in phosphate-buffered saline (PBS). Volumes (0.1 ml) were plated on Columbia Agar (Oxoid CM331) with 7% horse blood and on a selective medium. The latter comprised a nutrient base (Oxoid CM271) with the addition of horse blood 5% (v/v), brilliant green (G. T. Gurr Ltd) 1 in 60 000 and novobiocin (Upjohn Ltd, Crawley, Sussex) 5 µg per ml. The presence of brilliant green in solid media reduces the viable counts of *C. sputorum* subsp. *mucosalis* obtained whilst permitting the resistant colonies of this organism to grow freely. Stock solutions of brilliant green and representative batches of media were tested to confirm that the reduction in viable counts of broth cultures plated on the selective medium did not exceed 1 log₁₀ dilution in comparison with blood agar.

Plates were incubated at 37°C in a microaerophilic atmosphere (Lawson and Rowland, 1974) and examined daily. Total colony counts were recorded at the time representative

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subcultures were made. Subcultures from primary colonies were examined for pigment and in gram-stained smears, slide-catalase tests and slide-agglutination tests with *mucosalis* "OH" antisera. Yellow-pigmented, catalase-negative vibrios reacting in slide agglutination tests were considered to be *mucosalis* and the viable counts were estimated from these results. Representative strains from each site of isolation were then arbitrarily selected and examined in detail.

Strains were obtained from saliva by the method of Lawson *et al.* (1975b).

Electron microscopy. Tissues were prepared and examined as described by Rowland and Lawson (1974). Photographs of adenomatous cells were studied and bacteria showing evidence of division were recorded.

Biochemical tests

The method of testing for the ability of the organism to grow in the presence of inhibitory substances was the replicate-plating method used previously (Lawson *et al.*, 1975a). Tests for reduction of nitrate and nitrite, and for the production of catalase and hydrogen sulphide were also as described by Lawson *et al.* (1975a).

Serological tests

Antisera. The preparation of antisera in rabbits against whole-cell ("OH") vaccines has been described (Lawson and Rowland, 1974). Antisera were also prepared against autoclaved suspensions of organisms ("O"). These designations should not be taken to imply a correspondence with similarly named classes of antigens in other bacterial species. Bacteria for the preparation of O antisera were obtained from 48-h growth on Columbia-blood-agar slopes, washed once by centrifugation and resuspension in distilled water. The final suspension in distilled water was autoclaved for 1 h at 121°C, then centrifuged, and the deposit resuspended in NaCl 0.4% (w/v); merthiolate was added to a final concentration of 1 in 10 000. The injection schedule was as for the production of the OH antisera.

Agglutination tests. OH suspensions were prepared from 48-h cultures on Columbia-blood-agar slopes (Lawson and Rowland, 1974) and stored as concentrated antigen at 5°C in formal saline containing 0.12% formaldehyde. Bacteria for O suspensions were harvested in distilled water and centrifuged at 4000 r.p.m. for 30 min.; the deposit was resuspended in distilled water, held for 1 or 2 h at 75°C, or 100°C, or for 1 h at 121°C, cooled, centrifuged and resuspended in NaCl 0.4% (w/v). Immediately before use, the antigen preparations were adjusted to a standard density (approximately equal to that of Brown's opacity tube no. 2) by means of a nephelometer, the OH suspensions being diluted with NaCl 0.75% (w/v) and the O suspensions with NaCl 0.4% (w/v).

OH-agglutination tests were placed briefly in a 56°C water bath to ensure mixing before being incubated overnight at 37°C. O-agglutination tests were incubated at either 37°C or 56°C.

Alternative methods of preparing these antigens proved unsatisfactory; heat-treated formalinised antigens, and antigens treated with ethanol at 45°C were unstable in suspension. The preservation of O antigens with merthiolate appeared to reduce their reactivity progressively to O antisera.

Slide-agglutination tests. These were done with the surface growth from solid media suspended in saline and antisera preserved with glycerine 30% (v/v).

Absorption. After appropriate dilution with NaCl 0.75% (w/v), the serum was added to the absorbing antigen; the suspension was agitated for 1 h at room temperature and then centrifuged. Titrations of the absorbed and unabsorbed antisera were carried out in parallel.

Fluorescent staining. Goat anti-rabbit serum was purified by precipitation with rivanol (Mostratos and Beswick, 1969) and conjugated with fluorescein isothiocyanate (FITC) according to Nairn (1964). Saline suspensions of the organisms were air-dried on formalinised gelatin-treated slides and were stained by an indirect technique.

RESULTS

Quantitative studies in pigs

Enumeration of the organism is complicated by the fact that viable counts on media containing novobiocin and brilliant green are lower than counts on non-selective media. This occurs not only with laboratory cultures but also when bacteria are isolated directly from intestinal adenomatous tissue. It would be preferable to make counts of the organism on non-inhibitory media, but the advantages of selective media outweigh their disadvantages. Comparisons of the numbers of organisms in tissues were accordingly made from counts on selective media. These counts were invariably lower than counts obtained on non-inhibitory media but were within 1 log₁₀ dilution of the latter.

Although tissue dilutions were prepared in RCM, chyme was diluted in PBS (see *Methods*). Comparisons of dilutions of chyme in RCM and PBS from cases of porcine intestinal adenomatosis (PIA) indicated that these yielded comparable numbers of organisms.

In table I we present the results of quantitative studies on eight pigs with histologically confirmed intestinal adenomatosis. Irrespective of whether the lesion was in the mucosa of the small or large intestine, large numbers of *C. sputorum* subsp. *mucosalis* were isolated, and the number of organisms correlated well with the site of occurrence of the predominant mucosal lesion. When only the colonic mucosa was adenomatous, infection was restricted mainly to the affected tissue and the organism could not be isolated from the normal small-intestinal mucosa in two of such animals. When wall and chyme were sampled in parallel, the numbers of *mucosalis* present in the mucosa were invariably the greater. We were able to isolate the organism from some but not all of a small number of faecal samples.

TABLE I

Number of Campylobacter sputorum subsp. mucosalis isolated from pigs in relation to the presence of lesions of intestinal adenomatosis

Animal no.	Viable count* (per g wet weight) from					
	mid ileum: mucosa	lower ileum: mucosa	lower ileum: chyme	colon: mucosa	colon: chyme	faeces
614/73	...	...	...	3.20 × 10⁶	1.40 × 10 ⁵	...
5/74	...	3.80 × 10 ³	...	8.40 × 10⁶	2.10 × 10 ⁵	...
60/74	...	NR	...	2.16 × 10⁶	7.68 × 10 ⁵	NR
101/74	...	NR	...	5.36 × 10⁶	1.06 × 10 ⁶	8.60 × 10 ³
489/74	...	3.06 × 10 ³	...	2.46 × 10⁶	NR	NR
604/74	...	1.00 × 10 ⁵	...	2.96 × 10⁶	1.60 × 10 ⁵	...
632/74	6.20 × 10⁴	9.15 × 10⁴	...	...	...	...
106/75	NR	8.95 × 10⁶	4.45 × 10 ⁶	...	...	3.52 × 10 ⁶

* On blood agar containing novobiocin and brilliant green.

... = Not examined; NR = *mucosalis* not isolated.

In bold type: tissue grossly and histologically adenomatous.

TABLE II
Some properties of catalase-negative vibrios isolated from various pathological conditions in pigs, and from normal pigs

Pathological character of lesion or source	Animal no.	Number of isolates examined	Result of test for		Growth in the presence of					Agglutinability with OH antiserum in tube test†	
			nitrate reduction	nitrite reduction	H ₂ S*	glycine %		NaCl %			sodium deoxycholate %
						1.0	1.5	1.5	2.5		
PIA	252/72	1	+	+	+2	-	-	+	+	-	+
	253/72	1	+	+	+2	-	-	+	+	-	+
	302/72	1	+	+	+2	-	-	+	+	-	+
	5/74	3	+	+	+2	-	-	+	+	-	+
	60/74	2	+	+	+2	-	-	+	+	-	+
	101/74	2	+	+	+2	-	-	+	+	-	+
	489/74	2	+	+	+3	-	-	+	+	-	+
NE	604/74	3	+	+	+2	-	-	+	+	-	+
	124/73	2	+	+	+2	-	-	+	+	-	+
	614/73	1	+	+	+2	-	-	+	+	-	+
	182/74	1	+	+	+2	-	-	+	+	-	+
RI	1181/73	2	+	+	+2	-	-	+	+	-	+
	632/74	2	+	+	+2	-	-	+	+	-	+
Faeces†	9/74	1	+	+	+2	-	-	+	+	-	-
	20/74	2	+	+	+2	-	-	+	+	-	-
Faeces†§	1189/74	1	+	-	+2	-	-	-	-	-	-
	1192/74	1	+	+	+2	-	-	-	-	-	-
	59/75	2	+	+	+2	-	-	+	+	-	-
	106/75	1	+	+	+2	-	-	+	+	-	-
	207/75 A	1	+	+	+2	-	-	+	+	-	-
	207/75 B	1	+	+	+2	-	-	+	+	-	-
	207/75 C	1	+	+	+2	-	-	+	+	-	-
	207/75 C	1	+	+	+2	-	-	+	+	-	-

PIA = Porcine intestinal adenomatosis; NE = necrotic enteritis; RI = regional enteritis.

* = Hydrogen sulphide production in triple-sugar iron agar at 2 or 3 days.

† = Agglutination titre with 253/72 OH antisera ≥ 640 .

‡ = Clinical case of PIA.

§ = Typical strain later isolated from necropsy (no. 101/74).

|| = Not clinically diseased.

Intracellular division

The majority of bacterial profiles were incomplete or observed in transverse section. In sections from 12 pigs with intestinal adenomatosis, 225 full bacterial longitudinal profiles were present and clear evidence of septation was observed in three of these organisms (figure).

Characterisation of strains

Table II sets out the results of a series of laboratory tests on catalase-negative vibrios isolated from diseased and healthy pigs. The isolates from adenomatous tissue were a homogeneous group of organisms and were remarkably uniform when examined in the tests that had previously proved useful in identifying the subspecies. All the strains, whether isolated from cases of PIA, NE or RI, proved to have similar antigenic and biochemical properties. Typical *mucosalis* strains have also been isolated from the faeces of animals later shown to be affected with PIA. However, one strain of a catalase-negative vibrio, which differed antigenically and in tolerating higher concentrations of glycine and salt, was isolated from the faeces of an animal (no. 20/74) later shown to be affected with PIA; the same animal yielded a typical *mucosalis* strain from the affected mucosa at necropsy.

The population of mouth vibrios appears to be complex; many were biochemically identical with *mucosalis*, but others differed in their salt tolerance and ability to reduce nitrite. Whilst some mouth strains reacted with *mucosalis* OH antisera, and thus could not be differentiated from *mucosalis*, others failed to react at all in these tests. We should emphasise, however, that the predominant catalase-negative vibrios isolated from adenomatous lesions conformed to the description, both biochemically and antigenically, of *mucosalis* and that the slightly dissimilar ("atypical") organisms have not been isolated from the adenomatous lesions.

Agglutination studies with OH-antigen preparations in tubes. All three antisera prepared against the catalase-negative vibrios reacted to high titre with all the typical *mucosalis* strains tested. One serum was tested extensively and reacted with all the *mucosalis* strains, whilst failing to react at low dilution (20) with any of the 28 strains of catalase-positive vibrios tested. All three *mucosalis* antisera behaved similarly with the antigens tested and no attempt was made to test all strains with all the antisera. A series of eight antisera prepared against different types of catalase-positive *Campylobacter coli* (Lawson *et al.*, 1975a) failed to react with any of the catalase-negative organisms tested. Examination of the "atypical" catalase-negative strains confirmed the differences noted in other tests in that they failed to react with *mucosalis* antisera. During these tests, the control antigen regularly reacted with its homologous antisera with the end-point fluctuating between 1 in 2560 and 1 in 10 240. Antigens prepared from strains from a case of NE (no. 124/73) were observed in a number of tests to react consistently to lower titre than strains from other animals, and for this reason an antiserum was prepared against a selected strain (no. 124/73 B4) from this animal.

Agglutination studies with O-antigen preparations in tubes. When O-antisera were titrated against OH-antigen suspensions there was no agglutination with either the homologous strain, other *mucosalis* strains, or the catalase-positive vibrios. Exposure of the bacteria to heat at 75°C for 1 h reduced the OH agglutinability of the organism without inducing agglutinability with O-antisera. After exposure to 100°C for 1 or 2 h, suspensions became partly agglutinable by O antisera and also reacted with OH antisera. Agglutination of such suspensions occurs at greater dilution if the tests are performed at 56°C, but this was not our normal practice. Autoclaved suspensions reacted to high titre with homologous O antisera. These results are summarised in table III. All the *mucosalis* strains tested reacted with *mucosalis* O antisera, although there was some variation in the titre between different strains. Unlike the OH tests, O antigens of the catalase-positive *C. coli* strains did react with the *mucosalis* O antisera to a low titre (40–640). Attempts to characterise possible shared and specific components present have proved unsuccessful to date.

Slide-agglutination studies. The slide-agglutination tests confirmed the results of the tube agglutination tests. Undiluted O antisera did not react with young cultures of the *mucosalis* subspecies, although in older cultures some degree of agglutination may take place. OH antisera used in slide tests have never reacted with organisms that later proved atypical in tube tests, and OH antisera have never been observed to agglutinate strains that later proved to be catalase-positive vibrios.

Immunofluorescence studies. All the strains of the *mucosalis* subspecies tested have shown positive immunofluorescence with undiluted hyperimmune antisera. In contrast, only two of eight representative strains of *C. coli* showed

TABLE III
The effect of heat on the agglutinability of representative *mucosalis* strains

Treatment of OH suspension	Strain no.	Agglutination titre with antiserum		
		252/72 OH	253/72 OH	253/72 O
None	{ 253/72	5120	5120	< 20
	{ 302/72	5120	2560	< 20
75°C for 1 h	{ 253/72	2560	640	< 20
	{ 302/72	...	640	< 20
100°C for 1 h	{ 253/72	160	160	640
	{ 302/72	{ 160 320*	{ 160 320*	{ 640 ...
100°C for 2 h	{ 253/72	160	160	640
	{ 302/72	160	160	640
121°C for 1 h	{ 253/72	160	320	1280
	{ 302/72	320	1280	1280

* Test carried out at 56°C.

... = Not done.

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FIGURE.—Adenomatous epithelial cell containing intracellular bacteria, one of which shows septate division. Uranyl acetate and lead citrate. Electronmicrograph. $\times 60\,000$.

any evidence of fluorescence with undiluted antisera and this was lost when the serum was used at a dilution of 1 in 10. All the *mucosalis* strains tested reacted at a dilution of 1 in 20 and homologous strains reacted at a serum dilution of 1 in 80.

Absorption experiments. These experiments have demonstrated that the surface antigens of the subspecies are complex in character and that the reactivity of strains with a single unabsorbed antiserum is dependent on a number of antigenic factors, all of which need not be present for the antiserum to react at dilutions approaching its maximum titre.

Absorption has demonstrated that strain 252/72 has a surface factor shared by no. 302/72 but not present in no. 253/72. Similarly, strains 253/72 and 124/73 B4 have a factor not present in no. 302/72, and strain 124/73 B4 has a factor not present in the other three strains. A simplified antigenic analysis of these strains is as follows.

Strain 252/72	a	b	...	d	...
Strain 253/72	a	b	c	...	...
Strain 302/72	a	b	...	d	...
Strain 124/73 B4	a	...	c	d	e

A summary of the absorption experiments is given in table IV.

DISCUSSION

These studies confirm that organisms with essentially similar characteristics may be isolated from the affected intestinal mucosa of pigs with the conditions of PIA, NE or RI. The latter two conditions show varying amounts of the remaining mucosa to be adenomatous, and it has been suggested (Rowland and Lawson, 1975) that in certain cases PIA may precede either NE or RI.

TABLE IV

Serum titres before and after absorption of mucosalis antisera by various antigens

Antiserum prepared against strain no.	Absorbing strain no.	Titre obtained with absorbed serum against OH antigen of strain no.			
		253/72	252/72	124/73 B4	302/72
252/72 OH	NA*	5120	10 240	...	5120
253/72 OH	NA	5120	2560	640	2560
124/73 B4 OH	NA	5120	...	10 240	1280
252/72 OH	253/72	< 40	...	160	2560
253/72 OH	124/73 B4	5120	...	80	2560
253/72 OH	302/72	640	...	...	< 40
124/73 B4 OH	253/72	< 40	...	2560	...
124/73 B4 OH	253/72 } 302/72 }	< 40	...	5120	< 40
124/73 B4 OH	302/72	...	...	5120	< 40

NA = Unabsorbed; ... = Not done.

In pigs with PIA, the greatest concentrations of *C. sputorum* subsp. *mucosalis* occur in association with the adenomatous tissues; the areas of normal intestinal mucosa present in adenomatous cases do not harbour the organism, which is present in larger numbers in the affected tissue than in the intestinal lumen. We have no direct evidence to indicate that the intracellular vibrio brings about the adenomatous change. However, it seems possible that either (1) the epithelial cell is preferentially parasitised by the bacteria and that a change is subsequently induced in the gland cells, or (2) that adenomatous epithelium developing as a result of some other influence specifically phagocytoses this organism from a postulated luminal population. The first proposition is supported by the presence of dividing organisms within the cell cytoplasm and by the lack of morphologically similar bacteria adjacent to the surface of the adenomatous cell (Rowland and Lawson, 1974). A logical concept based on these observations is that immature epithelial cells become infected by the organisms, that these persist intracellularly by multiplying in the dividing cells, and that infection thus spreads within the epithelium.

The present studies confirm the presence of *C. sputorum* subsp. *mucosalis* and other catalase-negative vibrios in small numbers in the oral cavity of pigs. All the latter organisms differ serologically and some differ biochemically from *mucosalis* subspecies. We feel that at present nothing is to be gained by applying a specific epithet to these organisms and we retain the name *mucosalis* to identify the typical organisms isolated from adenomatous tissue, or serologically and biochemically identical organisms isolated from other sites.

Small but distinct antigenic differences exist between strains of the *mucosalis* subspecies. It is not clear at present whether these differences have any epidemiological significance or whether they merely indicate an antigenic shift related to host adaptation.

These results uphold and extend our opinion that the *mucosalis* strains form a distinct subspecies. One of our earlier strains has been deposited with the National Collection of Type Cultures as the neotype strain (*C. sputorum* subsp. *mucosalis* strain no. 253/72, no. NCTC11 000).

SUMMARY

Catalase-negative vibrios can be isolated in large numbers from the affected intestinal mucosa of pigs suffering from a range of porcine enteropathies in which the mucosa has an adenomatous component. These vibrios cannot be distinguished from strains of *Campylobacter sputorum* subsp. *mucosalis*. The resemblance between these bacteria strengthens the case already made on morphological evidence that these enteropathies have a common origin.

Catalase-negative vibrios have also been isolated from the mouth and faeces of pigs. Some of these conform to the criteria established for the *mucosalis* subspecies but others can be differentiated from it.

An antigenic analysis shows that strains of the *mucosalis* subspecies are closely related antigenically, but that differences may allow separation of strains.

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