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GRAM-NEGATIVE ANAEROBES IN THE INTESTINAL FLORA OF PIGS

By

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AALBÆK, BENT: *Gram-negative anaerobes in the intestinal flora of pigs.* Acta vet. scand. 1972, *13*, 228—237. — A differential count of the gram-negative, anaerobic, non sporeforming bacteria in different segments of the pig intestinal tract was performed in 2 groups of pigs. Seventy-seven strains were identified, belonging to 6 groups: Sphaerophorus necrophorus, anhemolytic Sphaerophorus spp., Bacteroides (Eggerthella) spp., Fusobacterium spp., Veillonella/Acidaminococcus spp., and Peptostreptococcus elsdenii. The characters of these groups are described, and quantitative data on their occurrence in a group of normal porkers and a group of pigs with experimental swine dysentery are given.

gram-negative anaerobes; Bacteroidaceae; threonine deaminase; intestinal flora; pigs; swine dysentery.

There have been several recent investigations on individual genera within the family Bacteroidaceae, e.g. on Fusobacterium (*Baird-Parker* 1960), Sphaerophorus (*Fievez* 1963), and Bacteroides (Eggerthella) (*Beerens et al.* 1963, *Werner* 1968). Among the recent investigators of the pig intestinal flora, few have undertaken a differentiation of the gram-negative anaerobic rods, numerously present. The aim of the present study was to procure further data on the identity and number of the Bacteroidaceae in the pig intestinal flora.

MATERIALS AND METHODS

From 3 normal porkers (approx. 6 months old, weighing 90 kg) and from 8 pigs with experimental swine dysentery (*Eriksen & Andersen* 1970), produced by feeding intestinal material from spontaneous cases to pigs, weighing about 30 kg, the entire intestinal tract was removed immediately after killing. After ligation, the following seg-

ments were removed: duodenum (about 15 cm, beginning from pylorus), jejunum (about 15 cm in the middle of jejunum), ileum (about 10 cm, terminating 2 cm from caecum), caecum (the apex), and colon dextrum (about 20 cm of the apex of the spiral coil of colon). In less than 1 hr. the segments were opened aseptically and 1 g of the intestinal contents was weighed into a tube, containing 9 ml freshly boiled and cooled VL broth, which was covered with vaseline until further dilutions up to 10^{-8} could be prepared. Surface cultures, representing 10^{-3} to 10^{-9} g of the samples were made by spreading 0.1 ml amounts of the dilutions on the media mentioned. The cultures were incubated anaerobically by the alcaline pyrogallol method at 37°C for 3—5 days. Morphologically identical colonies were counted, chiefly on VL blood agar (*Fievez* 1963), but to a necessary extent, search for specific organisms was also supported by use of the selective media: VL bile azide blood agar (*Beerens et al.* 1963) for Bacteroides (Eggerthella), VL brilliant green azide blood agar (*Fievez*) for Sphaerophorus, Fusobacterium medium (*Baird-Parker* 1960) for Fusobacterium, Rogosa medium (*Rogosa et al.* 1958) for Veillonella, and, for comparative purpose, also the selective medium of *Fuller & Lev* (1964). A representative for each morphological entity was isolated, and if being a gram-negative anaerobe, it was submitted to an identification procedure, using media and identification methods, previously used for Sphaerophorus necrophorus strains (*Aalbæk* 1971). For determination of threonine deaminase in some strains, 200 ml 16 hrs. VL broth cultures were centrifuged, and the cells were washed with 0.05 M tris buffer (pH 7.5) and suspended in 10 ml of the same buffer. The cells were disrupted by passing a french pressure cell. After centrifugation 0.5 ml supernatant was used as the source of enzyme. Threonine deaminase activity was demonstrated according to *Lessie & Whiteley* (1969) by following the production of apha-ketobutyrate at 30°C in assay mixtures, containing 2×10^{-1} M tris buffer (pH. 8.5), 4×10^{-2} M mercaptoethanol, 1.85×10^{-4} M pyridoxal phosphate, 0.5 ml enzyme preparation, and 10^{-1} M threonine (adjusted to pH 8.5) in a final volume of 1 ml. A blind test was added 1 ml 15 % trichloroacetic acid before enzyme was added. After 20 min. incubation, 1 ml 15 % trichloroacetic acid was added to the test solution. 0.2 ml of the mixture was mixed with 2 ml 0.02 % 2,4-dinitrophenylhydrazine in 0.4 N-HCl. After 20 min. at room temperature, 2 ml 2 N-NaOH was added and threonine deaminase activity was observed qualitatively by visual comparison of blind- and test solutions. For quantitative purpose the absorbance was measured at 450 nm.

RESULTS

The 77 strains isolated could be classed with 6 groups: Sphaerophorus necrophorus, anhemolytic Sphaerophorus spp., Bacteroides (Eggerthella) spp., Fusobacterium spp., Veillonella/Acidaminococcus spp., and Peptostreptococcus elsdenii. Their characters are given in Tables 1, 2, and 3.

Table 1. Cultural characters of 77 porcine intestinal strains of gram-negative anaerobes.

	Number of strains	Colony morphology*	Cell morphology**	Motility	Appearance of 24 hrs. Rosenow medium culture
Sphaerophorus necrophorus, type A	6	2.5 mm, low convex, coarsely granular, lobate margin	gram-negative rods, variable length with dominance of filaments	—	gas (large amounts), turbidity, red colour (acid), decolouration
Sphaerophorus necrophorus, type B	2	1 mm, pulvinate, yellowish, opaque	gram-negative rods, dominance of short rods with granules	—	gas (large amounts), flocular growth, red colour (acid), decolouration
Anhemolytic Sphaerophorus spp.	24	3 mm, convex, finely granular, lobate margin	gram-negative rods, extremely polymorphous, with dominance of protoplast-like forms	—	gas (large amounts), turbidity, red colour (acid), decolouration
Bacteroides (Eggerthella) spp.	9	1.5 mm, pulvinate, translucent, entire margin	gram-negative, uniform short rods with rounded ends and bipolar staining	—	gas (small amounts), turbidity, red colour (acid), no decolouration
Fusobacterium spp.	7	0.5 mm, convex, finely granular, entire margin	gram-negative, uniform rods with pointed ends	—	no gas, uncoloured
Veillonella/Acid-aminococcus spp.	18	0.5 mm, pulvinate, smooth, entire margin	small gram-negative cocci	—	gas (small amounts), uncoloured
Peptostreptococcus elsdenii	11	1 mm, pulvinate, yellowish, entire margin	large gram-negative cocci	—	gas (small amounts), uncoloured

* Colony morphology of surface cultures on VL blood agar (37°C/72 hrs.).
** Cell morphology of Rosenow medium cultures (37°C/24 hrs.).

Table 2. Biochemical characters of 77 porcine intestinal strains of gram-negative anaerobes.

	Number of strains	Hemolyses (horse blood)	Hemagglutination* (chicken erythrocytes)	Average pH in 1 % glucose broth cultures	Growth in 0.0008 % brilliant green**	Growth in 10 % bile**	Indole production	Nitrate reduction	Cysteine milk	Relative amounts of fatty acids, produced in broth***						Threonine deaminase****
										acetic acid	propionic acid	butyric acid	valeric acid	isovaleric acid	caproic acid	
Sphaerophorus necrophorus, type A	6	+	+	5.60	+	4(+) 2 —	+	—	pept.	+	+	+++				+
Sphaerophorus necrophorus, type B	2	+	—													
Anhemolytic Sphaerophorus spp.	24	—		5.17	+	+	2+ 22—	—	no change	+	+	+++				see text
Bacteroides (Eggerthella) spp.	9	+ (weak)		4.89	4 + 2(+) 3 —	++	—	—	coag.	++	+	(+)	(+)	+	(+)	—
Fusobacterium spp.	7	—			+	—	+	—	no change	+	+	++				+
Veillonella/Acid-aminococcus spp.	18	—					—	5+ 13—	no change							
Peptostreptococcus elsdenii	11	—		6.13	5 + 2(+) 4 —	—	—	—	no change	+	+	+	+	+	+	

* According to *Fievez* (1963).
** ++ : growth in test medium stimulated, compared to blind medium.
+ : growth in test medium equal to growth in blind medium.
(+): growth in test medium partially inhibited, compared to blind medium.
— : growth totally inhibited in test medium.
*** Demonstrated by gas-liquid-chromatography.
Increasing, relative amounts of fatty acids indicated by (+), +, ++, and +++.
**** Threonine deaminase, demonstrated according to *Suzuki et al.* (1966).

Table 3. Acid production from carbohydrates by 77 porcine intestinal strains of gram-negative anaerobes.

	Number of strains	Acid from*														
		arabinose	xylose	fructose	galactose	glucose	mannose	lactose	maltose	sucrose	cellobiose	melezitose	adonitole	sorbitole	mannitole	salicine
Sphaerophorus necrophorus, type A	6	—	—	+	6+	+	6+	—	6+	—	—	—	—	—	—	—
Sphaerophorus necrophorus, type B	2				2—		2—		2—							
Anhemolytic Sphaerophorus spp.	24	—	4+	+	+	+	+	18+	9+	17+	14+	1+	—	1+	11+	20+
			20—					6—	15—	7—	10—	23—		23—	13—	4—
Bacteroides (Eggerthella) spp.	9	—	7+	+	+	+	+	+	+	+	6+	—	—	—	1+	—
			2—								3—				8—	
Fusobacterium spp.	7	—	—	3+	—	3(+)	1+	—	—	—	—	—	—	—	—	—
				4—		4—	6—									
Veillonella/Acid-aminococcus spp.	18	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Peptostreptococcus elsdenii	11	—	—	10+	—	9+	6+	—	2(+)	—	—	—	—	—	—	—
				1(+)		1(+)	3(+)		9—							
						1—	2—									

* + : Acid production demonstrated after 4 days.
(+): Acid production weak and/or late.
— : Acid not produced during 14 days.

The threonine desaminase test of *Suzuki et al.* (1966) gave uniform results among the strains of Sphaerophorus necrophorus, Bacteroides, and Fusobacterium. Among the anhemolytic Sphaerophorus strains 50 % were found positive, but in a second experiment, only 33 % of the strains were found positive, several strains changing from + to — et vice versa. By prolonging the incubation period from originally 3 hrs. to 19 hrs. in a third experiment, the percentage of positive strains was increased to 75. For comparison these anhemolytic Sphaerophorus strains were also subjected to the nile blue test of *Beerens & Castel* (1965), in which 16 % were positive. Detection of threonine deaminase according to *Lessie & Whiteley* (1969) revealed the enzyme to be present in 100 % of the strains, however.

Quantitative data, indicating the average viable count of the 6 groups of gram-negative anaerobes identified from various intestinal segments of the porkers and the experimental dysentery pigs, are graphed in Fig. 1.

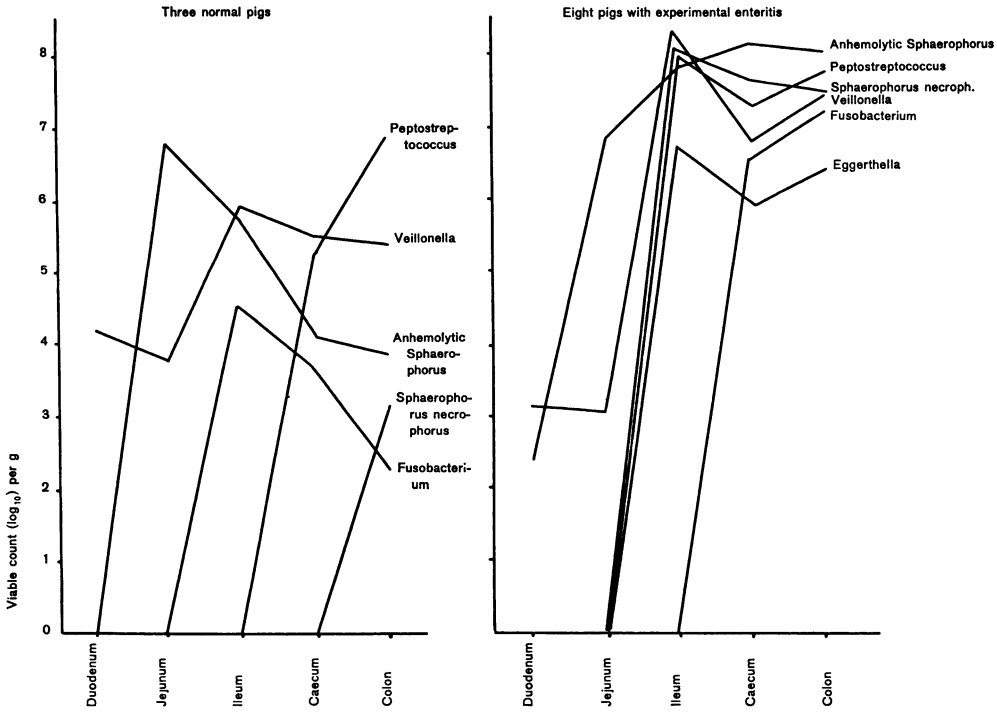

Figure 1. Average viable counts.

CONCLUSIONS

The characters of the group of anhemolytic Sphaerophorus strains agree with those reported by *Fievez* (1963). Complete agreement with species descriptions (*Prevot* 1966) is found in few strains, 1 strain having characters common with S. pseudonecrophorus, and 6 strains with S. necroticus. One strain has all characters common with those of "isolate 6-13-68" of *Pearson & Balish* (1970) from human feces.

Taxonomical importance has been given to the threonine metabolism in the Bacteroidaceae by the classification of *Beerens et al.* (1962), and screening tests have been developed for the characterization of strains (*Beerens & Castel* 1965, *Suzuki et al.* 1966). When applied to the present strains of anhemolytic Sphaerophorus, none of these tests were satisfactory, but reproducible results were obtained by using the test according to *Lessie & Whiteley* (1969) for demonstration of threonine deaminase.

After some years of doubt as to the justification of species division within the genus Bacteroides (Eggerthella) (*Beerens et. al.* 1963), the results of *Werner & Rintelen* (1968) seem to justify a species division similar to that of *Eggerth & Gagnon* (1933). Among the present strains, 5 are similar to B. fragilis, 3 to B. vulgatus, and 1 to B. incommunis.

Among the gram-negative small cocci, 5 nitrate reducing strains are comparable to the definition of *Rogosa* (1964) for Veillonella, whereas 13 strains, not reducing nitrate, are comparable to the definition of *Rogosa* (1969) for Acidaminococcus, a genus created for strains isolated from the pig intestinal tract by *Fuller & Lev* (1964).

The characters of the large gram-negative cocci are in good agreement with those described for Peptostreptococcus elsdenii by *Gutierrez et al.* (1959), except that most of the present strains produce acid from mannose.

As seen from Fig. 1., in the group of porkers the only gram-negative anaerobe found in duodenum was Veillonella/Acidaminococcus. In the jejunum, further anhemolytic Sphaerophorus were present in large numbers. In ileum further Fusobacterium, and in caecum Peptostreptococcus were present. As stated by *Fievez,* isolation of Sphaerophorus necrophorus from pigs intestinal flora is rarely successful. In the present study it was found in a small number in colon of 1 porker out of 3 examined.

Because of difference in age and diet, a comparison between the experimental swine dysentery group and the normal porkers should be made with reservation. In the dysentery group the number of all gram-negative anaerobes is much higher than in the porkers. Compared to the localization of the genera in the intestinal segments of the porkers, a forward movement is observed in the dysentery group. Bacteroides (Eggerthella), which was not demonstrated in the porkers, was present in large numbers in the enteritis group, and the most outstanding species was B. fragilis, which is unfrequent in the human intestinal flora, but frequent in pathological processes. Sphaerophorus necrophorus, known as a secondary invader after damage to the intestinal mucosa, was present in large numbers in the areas of enteritis.

DISCUSSION

The most outstanding representative of the Bacteroidaceae in the human intestinal flora is the genus Bacteroides (Eggerthella), and the most prominent species are B. vulgatus and B. thetaiotaomicron (*Werner et al.* 1970), whereas the majority of strains from human pathological material are B. fragilis. Conversely, one of the most outstanding representatives for the Bacteroidaceae in the porkers was the group of anhemolytic Sphaerophorus. This genus is not considered as a major component of the human intestinal flora (*Werner et al.*), but its presence in the fecal flora of 2 persons, receiving cobalt radiation therapy, has been reported (*Pearson & Balish* 1970).

The groups of gram-negative, anaerobic bacteria found in the porkers agree with the groups reported by *Fuller* (1966): group I, with some resemblance to Fusobacterium, group II, belonging to genus Sphaerophorus, group III, Veillonella, later regrouped as Acidaminococcus (*Rogosa* 1969, gen.nov.), and Peptostreptococcus. Bacteroides (Eggerthella) was not demonstrated, but the medium used, containing ethyl violet, would be inhibitive to this genus.

In the experimental swine dysentery group, the presence of large numbers of Sphaerophorus necrophorus is likely to represent a factor in the trend of pathological events, while the role of Bacteroides fragilis is unclear.

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SAMMENDRAG

Gramnegative anaerobe bakterier i svinets tarmflora.

Der udførtes differentialtælling af gramnegative, anaerobe, nonsporogene bakterier i forskellige afsnit af tarmkanalen hos 2 grupper svin. Der identificeredes 77 stammer, som kunne henføres til 6 grupper: Sphaerophorus necrophorus, anhæmolytiske Sphaerophorus spp., Bacteroides (Eggerthella) spp., Fusobacterium spp., Veillonella/Acidaminococcus spp. og Peptostreptococcus elsdenii. Egenskaberne hos disse grupper beskrives og deres forekomst hos en gruppe slagterisvin og en gruppe svin med eksperimentel svinedysenteri angives.

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