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MICROCOCCUS INDOLICUS

SOME BIOCHEMICAL PROPERTIES, AND THE DEMONSTRATION OF SIX ANTIGENICALLY DIFFERENT TYPES

By

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HØI SØRENSEN, GUNNER: *Micrococcus indolicus. Some biochemical properties, and the demonstration of six antigenically different types.* Acta vet. scand. 1973, *14*, 301—326. — A total of 274 strains of Micrococcus indolicus, 211 of which had been isolated from cases of summermastitis, 13 from other cases of mastitis in cattle, 15 from other suppurative lesions in cattle, 13 from insects, 13 from the vagina and interdigital skin of clinically healthy cows, and 9 from various suppurative lesions in swine, were studied and compared with 4 strains of anaerobic cocci of human origin, presumably representing at least 3 species closely related to Mi. indolicus: Staph. asaccharolyticus Distaso, Staph. aerogenes Schotmüller, and Staph. anaerobius Jungano.

The growth characteristics of Mi. indolicus are described, and the most important biochemical criteria for its identification stated briefly (Table 1).

By double diffusion-in-gel analysis 217 strains of Mi. indolicus could be divided in 6 antigenic types, designated A, B, C, D, E, and F (Figures 2, 3, 4, and 5, Tables 2, 3, 4, and 6).

By the complement fixation technique no difference could be demonstrated between the 6 types (Table 5).

Strains isolated from healthy cattle or from insects all belonged to antigenic types commonly found in summermastitis.

Three of 9 porcine strains belonged to a type (F) hitherto not found in cattle.

The 4 human strains of anaerobic cocci showed no antigenic relation to Mi. indolicus (Table 5) and differed from it in growth characteristics and in biochemical properties (Table 1).

It is concluded that, as suggested by *Christiansen* (1934) Micrococcus indolicus should be classified as a species of its own and not as a variant of Staphylococcus asaccharolyticus Distaso (*Prévot et al.* 1967).

micrococcus indolicus; anaerobic cocci; summermastitis; staphylococcus asaccharolyticus distaso.

Micrococcus indolicus was first isolated from 2 cases of chronic, suppurative thelitis and galactophoritis in young calves (*Christiansen* 1934).

A similar anaerobic coccus, associated with Corynebacterium pyogenes, was demonstrated by *Leth Jørgensen* (1937), and by *Prévot & Thouvenot* (1954) (syn. Staphylococcus asaccharolyticus var. indolicus, Christiansen) in secretions from cases of acute or chronic mastitis in cows.

Mi. indolicus has regularly been isolated from cases of summermastitis, usually associated with one or more of the following three organisms: Cb. pyogenes, Streptococcus dysgalactiae, and an unclassified microaerophilic coccus, but sometimes in monoculture (*Stuart et al.* 1951, *Cornelisse et al.* 1970 (syn. Peptostreptococcus indolicus) and *Høi Sørensen* 1973).

Little is known about the possible association of this organism with other suppurative conditions in domestic animals, though it has been isolated from cases of metritis and traumatic reticulitis in cattle (*Stuart et al.*) and from various cases of suppurative inflammation a priori diagnosed as cases of Cb. pyogenes infection, such as cases in cattle of arthritis, bursitis, otitis media, endometritis, abortion, and abscesses, and cases in swine of endometritis, pericarditis, and abscesses (*Høi Sørensen* 1973).

Mi. indolicus has also been isolated from the vagina and interdigital skin of clinically healthy cows (*Høi Sørensen* 1973).

MATERIAL AND METHODS

The present study comprises a total of 274 strains of Mi. indolicus originating from the following sources:

Cases of summermastitis occurring in 5 different areas of Jutland, 1966—1967	: 211 strains
Other cases of mastitis	: 13 strains
Insects	: 13 strains
Various suppurative conditions in cattle	: 15 strains
Vagina and interdigital skin of clinically healthy cows	: 13 strains
Various suppurative conditions in swine	: 9 strains

For details about sources, isolation, and purification of cultures, reference is made to *Høi Sørensen* (1973).

For comparison, 4 strains of anaerobic cocci from human sources were studied (reference strains 2229, 2480, 4598, and 5106). These strains, classified as Staphylococcus asaccharolyticus Distaso, were received from the Institut Pasteur, Paris.

Media

Solid

Blood agar: Meat infusion peptone agar (Bacto Peptone 1 %, Davis agar 1.2 %) with 5 % citrate-stabilized calf blood. pH 7.2—7.4.

Deep agar: as blood agar, but without calf blood. pH 7.2—7.4.

Serum gelatine: Meat infusion serum gelatine (Bacto Gelatine 15 %, horse serum 10 %). pH 7.4—7.6.

Ferrous sulphate agar: see *Jepsen* (1960).

Swarm agar: see *Kauffmann* (1951).

Liquid

Nutrient broth: Meat infusion peptone broth (Bacto Peptone 1 %). pH 7.2—7.4.

Beef-heart broth (Trypsin-digested beef-heart broth), casein broth (Trypsin-digested casein broth), beef-brain broth, Robertson's cooked-meat medium, nitrate broth, citrate medium, and basic medium for fermentation tests: see *Jepsen.*

Urea broth: see *Kauffmann.*

Incubation temperature

Unless otherwise stated cultures were incubated at 37°C.

Anaerobic incubation

was carried out in "BTL ANAEROBIC JARS" with hydrogen and 10 % carbon dioxide.

Storage of strains

For short-term storage 48-hour cultures in beef-brain broth were kept at room temperature. For prolonged storage 48-hour cultures on blood agar were suspended in skim milk and freeze-dried.

Microscopy and biochemical examinations

All the 274 strains of Mi. indolicus and the 4 strains of anaerobic cocci from human sources were subjected to the following examinations.

Microscopy

of Gram-stained films from 24-hour beef-heart broth cultures.

Indole test

Five-ml volumes of casein broth (in tubes approx. 1 cm in diameter and with a 1-cm overlay of liquid paraffin) were seeded with organisms grown for 24 hrs. in beef-heart broth. The casein-broth tubes were steamed for 30 min. shortly before being inoculated.

After incubation for 48 hrs. 2 ml were removed and tested for indole as indicated by *Jepsen*.

Hydrogen sulphide test

Tests for production of hydrogen sulphide were made on surface cultures on blood agar, on stab cultures in ferrous sulphate agar, and on cultures in nutrient broth, beef-heart broth, beef-brain broth, and Robertson's cooked-meat medium.

In cultures other than ferrous sulphate agar cultures, lead-acetate-impregnated filter-paper strips were used as indicator.

Plasma coagulase test

was carried out as indicated by *Jepsen* on citrate- or oxalate-stabilized calf and rabbit plasma.

Production of gas

Beef-brain broth was inoculated, at the bottom of the tube, with 24-hour nutrient broth culture and incubated aerobically.

The cultures were examined for gas production after 17 and 48 hrs. If the result of the test was doubtful the strain was retested in deep agar cultures, which were incubated for 48—72 hrs.

Catalase test

Colony material from blood agar was placed in a drop of 30 % hydrogen peroxide. Immediate bubbling was regarded as a positive reaction.

Twenty strains of Mi. indolicus, including the 6 reference strains mentioned below and the 4 human strains of anaerobic cocci, were in addition subjected to the following tests.

Nitrate reduction

Ten-ml volumes of nitrate broth inoculated with 24-hour blood agar culture were incubated anaerobically. Reading after 4 and 6 days. Tubes with uninoculated medium and tubes with inoculated basic medium without nitrate served as controls.

After 4 and 6 days the cultures were tested for nitrate with the reagent indicated by *Cruickshank* (1965) and for ammonia with Nessler's reagent.

Urease activity

Five-ml volumes of urea broth were inoculated with 24-hour blood agar cultures and incubated anaerobically for 4 days. Tubes with inoculated basic medium without urea served as controls.

Fermentation of carbohydrates

To basic medium 0.5 % of the test substances was added. Bromthymol blue served as pH indicator. Tubes with 5-ml volumes of medium

were incubated anaerobically for 24 hrs. prior to inoculation. Each tube was then inoculated with 4 drops of 20-hour nutrient broth culture and incubated anaerobically for 2, 4, 7, 14, and 21 days.

Liquefaction of serum gelatine

Tubes with 5 ml medium were inoculated with 24-hour-old cultures on blood agar and incubated anaerobically for 72 hrs. Before reading, the tubes were placed in refrigerator for 1 hr.

Sensitivity to antibiotics

Rosco sensitabs were placed on the surface of blood agar plates seeded with 24-hour nutrient-broth culture, and the plates incubated anaerobically. Results were read after 48 hrs., except in the case of the 4 human strains, which were incubated for 5 days before reading.

Optimum temperature for growth

Growth on blood agar was examined at 5, 11, 22, 37, and 44.5°C. The plates were pre-incubated for 24 hrs. at the respective temperatures and thereafter inoculated from 24-hour blood agar cultures. The plates were examined for growth intensity after 1, 2, 3, and 5 days.

Heat resistance

Organisms grown on blood agar were suspended in saline to a density of approx. 10^9 cells per ml (McFarland scale). One ml of each suspension was transferred to a tube with a diameter of approx. 1 cm and heated momentarily to 85°C on a water bath, immediately refrigerated in iced water, and inoculated deep in agar tubes. The tubes were incubated for 5 days.

Serological investigations

Preparation of antisera

Rabbits were immunized by intravenous injections of cells from 24-hour nutrient-broth cultures. The cells were washed 3 times and suspended in formalinized saline (0.2 % formalin, approx. 10^9 cells per ml according to the McFarland scale). A prolonged course of immunization (4—12 months) was necessary to produce antisera of sufficient potency for double diffusion-in-gel analysis.

Double diffusion-in-gel analysis

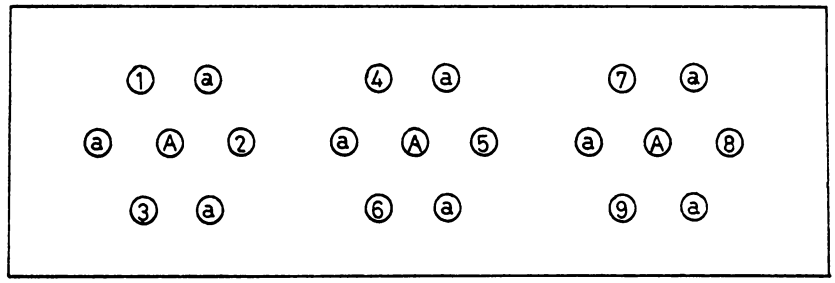
Preparation of bacterial extracts. The method of *Lancefield* (1933) for extraction of group-specific polysaccharides from streptococci proved unsatisfactory in the case of Mi. indolicus. The following method (*Vogel* 1954, *Lowell & Randall* 1955) was used instead:

Twelve to 15 ml of 24-hour beef-heart broth culture was centrifuged (Gerber centrifuge), the supernatant discarded, and the deposit suspended in 0.75 ml distilled water (WHIRLMIXER). After autoclaving at 120°C for 15 min., rapid release of the pressure, and cool-

ing in running tap water, the suspension was vigorously shaken on a WHIRLMIXER and centrifuged at 3000 r.p.m. for 30 min. The resulting supernatant was used as precipitating antigen.

Immune precipitation was performed as a micro-double-diffusion plate test in a gel prepared with 1 % AGAROSE BEHRINGWERKE in PBS of low ionic strength, pH 7.2 (*Olitzki* 1959). The gel was preserved with merthiolate 1:10.000.

Figure 1. Arrangement of antigen and antibody wells for double diffusion-in-gel analysis.



A = antiserum.
a = extract of homologous strain.
1—9 = extracts of heterologous strains.

Agarose solution (2.5 ml) was poured on a microscope slide placed in an exactly horizontal position. After solidification, 21 wells (diameter 2 mm) were punched in each plate in the pattern shown in Fig. 1. The volume of each well was 10 µl and the center-to-center distance between the wells in each group of 7 was 6.5 mm. An extract of each of the strains studied was tested against antisera for each of the 6 reference strains mentioned below.

The specific antisera were deposited in the center wells and the cell extracts of homologous strains in the surrounding wells as shown in Figure 1. In this way the precipitin band patterns of 9 strains with respect to 1 reference antiserum could be studied on each plate.

Two hundred and seventeen strains of Mi. indolicus (164 from cases of summermastitis, 53 from other sources) and 4 human strains of anaerobic cocci were examined.

Complement fixation test

Antigen for the complement fixation test was prepared as follows: Forty-eight-hour cultures in nutrient-broth were centrifuged and the deposits suspended in ⅓ vol. PBS, pH 7.4. After autoclaving for 20 min. at 120°C, rapid release of the pressure and cooling in running tap water, the suspensions were vigorously shaken and thereafter adjusted to a density of approx. 6×10^9 cells per ml (McFarland scale). Storage at —18°C.

Antigens were prepared in this way from the 6 reference strains of Mi. indolicus mentioned below and from the 4 human strains of anaerobic cocci. The complement-fixing power of the 6 Mi. indolicus antigens was titrated against their homologous antisera.

The complement fixation test. The test was performed by the technique given by *Jensen* (1956) except that serum doses 0.2—0.1—0.05 ml etc. were used instead of 0.18—0.09—0.045 ml etc., and that 5 titer doses of antigen were used instead of 2. Of complement 1.5 titer doses were used. The titer of a serum was defined as the smallest fraction of a ml in which it would produce a 100 % fixation of the complement, and indicated as the reciprocal of that fraction.

Pathogenicity for mice and guinea pigs

Mice and guinea pigs were inoculated i.p. with 0.2 ml doses of a 24-hour nutrient-broth culture of each of the 6 reference strains mentioned below, and observed for 3 months after the inoculation.

RESULTS

I. Micrococcus indolicus

Cell morphology and growth characteristics

Gram-stained films showed relatively small Gram-positive cocci, occurring singly or in pairs, short chains, tetrades, or small clusters.

All the 274 strains were strictly anaerobic, and growth seemed to be increased when, besides hydrogen, 10 % carbon dioxide was added to the jars.

After 24 hrs., colonies on blood agar were of pin-point size. Seen through a magnifying glass they were glistening and translucent though sometimes with an opaque center. After 48 hrs., colonies were circular, with entire edges, convex or cone-shaped, 0.5—0.75 mm in diameter, and greyish-yellow with a glistening surface. In older cultures the colonies reached a maximum diameter of 1.5—2.0 mm, but did not change essentially in shape and colour. No hemolysis was produced.

The consistence of the colonies varied widely. Some were butyrous and easily emulsified, others were viscid, and occasionally friable colonies occurred which would form granular suspension in saline. When subcultured some strains were rather constant as to colony consistence, while other strains were variable in this respect. The size and consistence of surface colonies were to some extent dependent on the humidity of the medium. The growth was most abundant and mucoid or viscid colonies

more frequent when freshly prepared medium was used, while the growth was more sparse and granular colonies easier formed, when the plates had been stored for more than 1 week.

The smell of 24-hour cultures was often slightly sourish, while older cultures always were of a markedly putrid odour.

In deep agar cultures no growth occurred in the upper 1—2 cm of the medium. Below this level there was uniformly good growth in the rest of the medium. The colonies were lenticular, greyish-yellow, and reached a maximum diameter of 1—2 mm after 4—5 days of incubation. Gas, disrupting the medium, was produced after 1—3 days.

In stab cultures in swarm agar, incubated anaerobically, growth was filiform, and motility was not observed.

All strains grew abundantly in the fluid media employed. Often a heavy turbidity and a viscid greyish-yellow sediment was formed. Some strains formed granules or floccules which settled at the bottom of the tubes; such cultures appeared but slightly turbid. Abundant gas was produced, and the odour was markedly putrid.

Growth was optimal at 37°C. At 22° and 44.5°C there was little if any growth, and at 5° and 11°C there was no growth at all. None of the 20 strains examined survived momentary heating at 85°C.

Biochemical reactions (Table 1)

All the 274 strains produced indole, gas, and hydrogen sulphide, and 267 were coagulase-positive. All were catalase-negative.

Indole-production was a prominent feature. An abundance of gas was produced by most strains after 24 hrs. A few strains produced no gas for the first 2 or 3 days, but after that they, too, produced it in abundance.

The production of hydrogen sulphide was very much dependent on the culture medium employed. Thus, 48—72-hour cultures on blood agar generally produced just a slight browning of the lead-acetate paper, and a distinct blackening was rarely seen. Cultures in beef-brain broth were negative or weakly positive after 48 hrs., while 48-hour cultures in nutrient broth and in beef-heart broth might be strongly positive or negative. Stab cultures in ferrous-sulphate agar always showed negative

Table 1. Biochemical properties and sensitivity to antibiotics of Micrococcus indolicus and 4 strains of anaerobic cocci of human origin.

	Indole	Gas	H_2S	NO_3/NO_2 reduction	Urea decomposition	Citrate utilization	Catalase	Coagulase	Glucose fermentation	Sorbitol	Trehalose	Penicillin	Streptomycin	Polymyxin-B	Neomycin	Terramycin	Aureomycin	Bacitracin	Chloramphenicol
Mi.ind.	+	+	+	+	0	0	0	+	0	0	0	S	R	V	V	S	S	S	S
Str.2229	(+)	+	+	+	0	0	0	0	0	0	0	R	R	R	S	S	S	S	S
Str.2480	+	+	+	(+)	0	0	0	0	+	(+)	+	S	S	R	S	S	S	S	S
Str.4598	(+)	0	(+)	0	0	0	0	0	0	0	0	S	R	R	R	R	R	R	S
Str.5106	0	0	(+)	0	0	0	0	0	0	0	0	S	S	R	S	S	S	S	S

(+) = faintly positive
S = sensitive
R = resistant or relatively resistant
V = variable

reaction even after prolonged incubation (5 days), while cultures in Robertson's cooked-meat medium regularly produced a distinct blackening of the lead-acetate paper after 17 hrs.

Tested on blood agar by the cup method, sodium thiosulphate, in the concentration (0.03 %) in which it is present in ferrous-sulphate agar, was found to have a markedly inhibiting effect on the growth of Mi. indolicus. Ferrous sulphate, of which there is 0.02% in the medium, has a slightly inhibiting effect in that concentration.

Coagulase for rabbit plasma was produced by 267 strains, and 263 of these also coagulated calf plasma, whereas 7 strains repeatedly were found to coagulate neither rabbit nor calf plasma.

Coagulase activity could be demonstrated in whole cultures as well as in culture filtrates (Millipore 0.22 μm) and saline suspensions of 3-times washed cells. The property seemed mainly to be cell-bound, since with whole cultures or cell suspensions positive reactions regularly appeared after 1—4 hrs., but with culture-filtrates not until the next day.

Twenty strains examined for nitrate reduction were all positive. After 4 days nitrate was reduced to nitrite or further on to ammonia.

None of the 20 strains decomposed urea or liquefied serum gelatine, and none of the following carbohydrates were fermented:

Saccharides	:	glucose, maltose, lactose, saccharose, trehalose, raffinose
Sugar alcohols	:	mannitol, sorbitol
Glycosides	:	salicin, aesculin

No growth occurred in citrate medium.

Sensitivity to antibiotics (Table 1)

All the 20 strains examined were sensitive to Penicillin, Bacitracin, Terramycin, Aureomycin, and Chloramphenicol, and all were relatively resistent to Streptomycin.

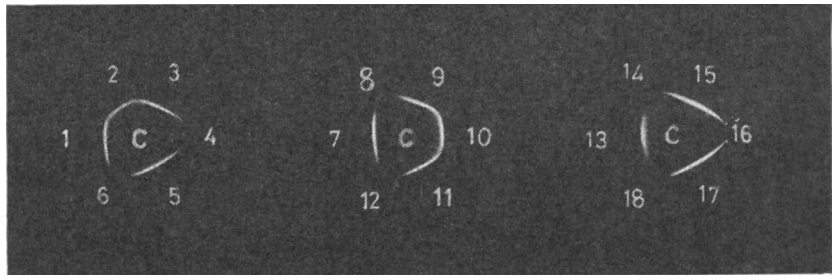
Five strains were sensitive to Neomycin and 2 to Polymyxin B. The remaining strains were relatively resistent to these 2 antibiotics.

Serological investigations

1. *Double diffusion-in-gel analysis*

Antisera were prepared against the following 6 strains of Mi. indolicus: R3, R8, R13, R14, and R33, all isolated from sum-

Figure 2. Double diffusion-in-gel analysis. The precipitin-band patterns of strain R13 and 9 heterologous strains.

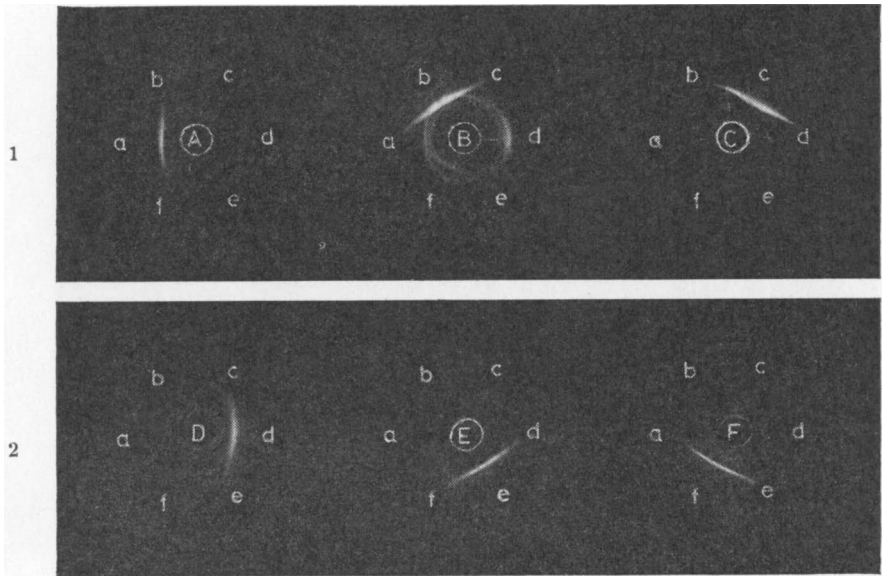


Center well (C): rabbit (No. 136) anti-R13 serum.
Wells 1, 3, 5, 7, 9, 11, 13, 15, and 17: Strain-R13 antigen.
Wells 2, 4, 6, 8, 10, 12, 14, 16, and 18: antigens of, respectively, Strains R66, R67, R68, R69, R70, R71, R72, R74, and R75.
Pattern of fusion ("reaction of identity") is seen at the junctions of the precipitin arches of Strains R66 and R70 (wells 2 and 10, resp.) with the adjacent Strain-R13 arches.

mermastitis exudates, and Gr.31, isolated from an intraperitoneal abscess in a pig.

Initially, rabbits were immunized with strain R13 and the resulting antisera tested in double diffusion tests against autoclaved extracts of this strain. When satisfactory precipitating titres were obtained, the antisera were tested against extracts of 50 strains, including the homologous Strain R13 (Fig. 1). As these strains turned out to be antigenically heterogeneous (Fig. 2) antisera were prepared against one of the strains which showed negative reaction with R13 antiserum, and these antisera were

Figure 3. Precipitin bands of the 6 immunological systems. The 6 type sera tested in double diffusion-in-gel against extracts of the 6 reference strains.



Center wells:

Slide 1: A: rabbit (No. 119) anti-A serum
B: rabbit (No. 95) anti-B serum
C: rabbit (No. 136) anti-C serum

Slide 2: D: rabbit (No. 120) anti-D serum
E: rabbit (No. 141) anti-E serum
F: rabbit (No. 135) anti-F serum

Surrounding wells:

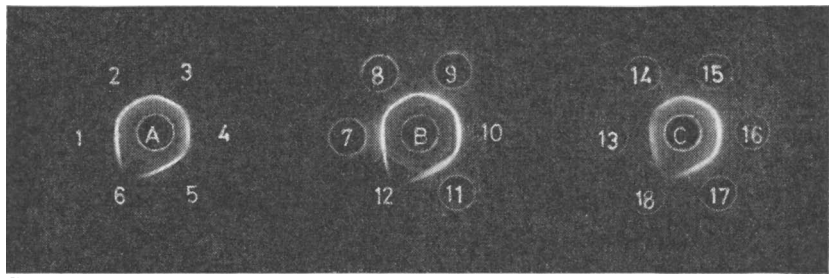
a, b, c, d, e, and f: extracts of corresponding reference strains, i.e., R3, R8, R13, R14, R33, and Gr.31, respectively.

tested against extracts of all the 50 strains. By continuing this procedure the 50 strains could finally be classified in 6 antigenically different types, designated A, B, C, D, E, and F, having reference to Strains R3, R8, R13, R14, R33, and Gr.31, respectively. Reactions between the 6 type antisera (anti-A to anti-F) and their homologous strains (the type strains) are shown in Fig. 3.

Finally, extracts of 217 strains of Mi. indolicus were tested against the 6 type sera, together with extracts of the 6 type strains. On the basis of precipitin band patterns all these strains could be referred to one or other of the 6 types.

Examples of reactions of identity, reaction of partial identity, and no reaction are shown in Figures 4 and 5. The 6 immuno-

Figure 4. Double diffusion-in-gel analysis. Strains of different origin tested against anti-A, anti-B, and anti-C serum. Two strains showing patterns of fusion and 1 strain showing no reaction or pattern of partial intersection are compared to each immunological system.



Center wells: A: rabbit (No. 119) anti-A serum
B: rabbit (No. 95) anti-B serum
C: rabbit (No. 136) anti-C serum

Surrounding wells, with extracts of strains:

Wells 1, 3, and 5 : R3 (homologous strain)
2 and 4 : R48 and R52 (classified as group A)
6 : R27 (no reaction)

Wells 7, 9, and 11 : R8 (homologous strain)
8 and 10 : U13 and U20 (classified as group B)
12 : U21 (partial intersection)

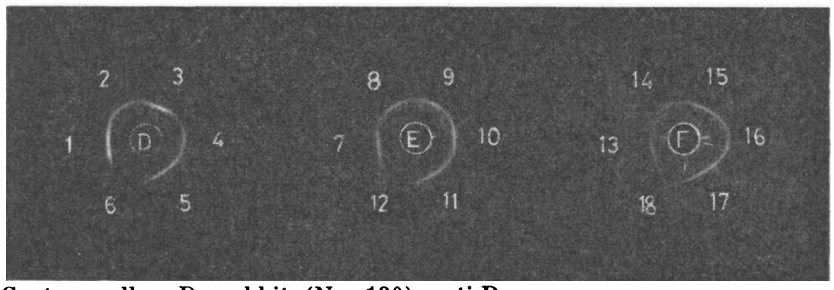
Wells 13, 15, and 17 : R13 (homologous strain)
14 and 16 : Ø29 and Ø37 (classified as group C)
18 : Ø28 (no reaction)

Strains designated R, U, and Ø were isolated from cases of summermastitis occurring near Rønde, Ulfborg, and Ølgod, respectively .

Table 2. The frequency of the 6 antigenic types in the total material studied.

Type	Number of strains
A	3
B	40
C	79
D	48
E	44
F	3
Total	217

Figure 5. Double diffusion-in-gel analysis. Strains of different origin tested against anti-D, anti-E, and anti-F serum. Two strains showing patterns of fusion and 1 strain showing no reaction are compared to each immunological system.



Center wells: D: rabbit (No. 120) anti-D serum
E: rabbit (No. 141) anti-E serum
F: rabbit (No. 135) anti-F serum

Surrounding wells, with extracts of strains:

Wells 1, 3, and 5 : R14 (homologous strain)
2 and 4 : S9 and S23 (classified as group D)
6 : S10 (no reaction)

Wells 7, 9, and 11 : R33 (homologous strain)
8 and 10 : R51 and FR33 (classified as group E)
12 : R58 (no reaction)

Wells 13, 15, and 17 : Gr.31 (homologous strain)
14 and 16 : Gr. 1 and Gr.32 (classified as group F)
18 : Gr.10 (no reaction)

Strains designated S and R were isolated from cases of summermastitis occurring near Sunds and Rønde, respectively. Strain designated FR was isolated from Hydrotaea irritans var. Fallén, collected and submitted together with the secretion from which Strain R33 was isolated. Strains designated Gr. were isolated from abscesses in swine.

logical systems are arranged on 2 slides, and 3 heterologous strains are tested against each system.

Most of the 217 strains fell within Type B, C, D or E, Type C occurring with the highest frequency, while but 3 strains were classified as Type A and another 3 as Type F (Table 2).

The precipitin-type distribution of strains originating from cases of summermastitis is shown in Table 3, and that of strains of other origin in Table 4. It should be added that it was not uncommon to find different (up to 3) types represented among strains originating from the same herd.

Table 3. Serotyping of strains isolated from cases of summermastitis occurring in 5 different areas of Jutland.

Strains originating from	Serotype A	B	C	D	E	F	Total of strains
Rønde	3	17	17	17	19	0	73
Sunds	0	3	27	14	5	0	49
Ulfborg	0	4	4	4	3	0	15
Ølgod	0	4	6	1	7	0	18
Ålborg	0	2	4	1	2	0	9
Total	3	30	58	37	36	0	164

Table 4. Serotyping of strains isolated from sources other than summermastitis.

Origin of strains	Serotype A	B	C	D	E	F	Total
insects	0	2	2	1	5	0	10
acute or chronic mastitis, cows	0	4	7	0	2	0	13
other suppurative lesions, cattle	0	0	3	4	1	0	8
clinically healthy cows	0	0	7	6	0	0	13
suppurative lesions, swine	0	4	2	0	0	3	9
Total	0	10	21	11	8	3	53

The 13 strains from healthy animals originated from 7 cows in 1 herd, and were isolated from samples taken from the vagina and interdigital skin at approximately monthly intervals over a period of 5 months. Types C and D were demonstrated on up to 4 occasions in 3 cows each (Table 6). In none of the cows was more than 1 type found.

Table 5. Complement fixation test. The 6 Micrococcus indolicus type sera tested against the 6 reference antigens and antigens of the 4 human strains of anaerobic cocci (Strains 2229, 2480, 4598 and 5106).

Rabbit antiserum	Antigen									
	R3(A)	R8(B)	R13(C)	R14(D)	R33(E)	Gr.31(F)	2229	2480	4598	5106
119 : anti-A	320	160	160	160	160	80	—	—	—	—
95 : anti-B	1280	1280	640	640	640	640	—	—	—	—
97 : anti-C	160	80	160	80	80	80	—	—	—	—
120 : anti-D	320	160	160	160	160	320	—	—	—	—
98 : anti-E	320	160	80	160	160	80	—	—	—	—
121 : anti-F	160	80	80	80	160	80	—	—	—	—

Figures indicate reciprocals of smallest amounts (in ml) of antiserum giving 100 % fixation.
— : no fixation with 0.2 ml serum.

Table 6. Serotyping of strains isolated from the vagina and interdigital skin of 7 clinically healthy cows (5 samplings at approximately monthly intervals).

Cow no.	Number of samples with Type C		Number of samples with Type D	
	vagina	interdig. skin	vagina	interdig. skin
1	0	0	3	1
2	0	0	1	0
4	0	0	0	0
5	1	2	0	0
8	2	1	0	0
13	1	0	0	0
26	0	0	0	1

2. Complement fixation test

As appears from Table 5, each of the type sera was found to contain complement-fixing antibodies for its homologous strain as well as for the other 5 type strains of Mi. indolicus. No differences between the 6 precipitin types could be demonstrated by cross complement-fixation test.

Pathogenicity for mice and guinea pigs

The mouse inoculated with Serotype E (Strain R33) died after 2 months. Post-mortem examination showed emaciation, some serous exudate in the peritoneal and pleural cavities, and hyper-

trophy of the suprarenal glands. Cultivation from spleen, liver, lungs, kidneys, exudates, and suprarenal glands gave no growth of Mi. indolicus. The guinea pigs and the mice inoculated with the other 5 serotypes showed no clinical signs of disease during the period of observation (3 months) and no pathologic changes post mortem.

II. The 4 strains of anaerobic cocci of human origin

Films of 48-hour cultures showed Gram-positive or Gram-labile cocci, while older cultures were regularly Gram-labile. They were strictly anaerobic, and grew considerably slower on solid media than Mi. indolicus. To obtain satisfactory growth in liquid media heavy inoculation from 4—5-day blood agar cultures was necessary.

On blood agar, Strains 2229, 2480, and 5106 formed translucent, glistening, non-hemolytic colonies, which were of pinpoint size after 3 days and reached a maximum diameter of ab. 0.25 mm after 7 days. Colonies of Strain 4598 were, after 3 days, non-hemolytic, 0.25—0.5 mm in diameter, circular with entire edges, low-convex, translucent (possibly with a greyish-opaque center) and glistening; a maximum diameter of approx. 0.75 mm was reached after 7 days.

Colonies of all strains were of a butyrous consistence and easily emulsified in saline.

In deep agar very delicate, scarcely visible colonies were formed after 48 hours. After 7—10 days colonies of Strain 4598 were lenticular, greyish-yellow, with a maximum diameter of ab. 0.75 mm. Colonies of the other 3 strains were less than 0.5 mm in diameter, slightly greyish, lenticular. No growth occurred in the upper 1—2 cm of the medium.

In deep agar medium enriched with 10 % horse serum, Strains 2229 and 2480 produced abundant gas, which would sometimes split the medium after only 24 hours. Strains 4598 and 5106 produced gas neither in plain nor in enriched medium. In general, addition of serum to the medium had a markedly promoting effect on the growth of all the 4 strains. In liquid media Strains 2229, 2480, and 4598 produced turbidity and a slightly viscid sediment. The growth of Strain 5106 was granular without turbidity, the granules rapidly settling at the bottom of the tube. All cultures had an unpleasant odour, which was different from that of Mi. indolicus.

Strains 2229, 2480, and 4598 were indole-positive, and hydrogen sulphide was produced by all of the 4 strains. However, in spite of heavy inoculation and prolonged incubation the indole and H_2S reactions were often very weak (Table 1). Nitrate was reduced to nitrite by Strains 2229 and 2480, but not by Strains 4598 and 5106. Strain 2480 fermented glucose, trehalose, and sorbitol, the other 3 strains not. All the 4 strains were catalase- and coagulase-negative, and did not utilize citrate. Urea was not decomposed, and serum-gelatine not liquefied.

The results of the biochemical tests and the tests for sensitivity to antibiotics are shown in Table 1.

None of the 6 Mi. indolicus type sera cross-reacted with any of the 4 human strains in gel analysis or complement fixation tests (Table 5).

DISCUSSION

I. Micrococcus indolicus

Since the first description of Mi. indolicus by *Christiansen* (1934), and later by *Leth Jørgensen* (1937), only few works have been published about this organism. This, in connection with insufficient taxonomic criteria for the anaerobic cocci and differing opinions about the nomenclature may explain why in international textbooks and manuals Mi. indolicus is not described as a well-defined species, if mentioned at all. In the sixth edition of *Bergey's Manual of Determinative Bacteriology* (1948), Mi. indolicus is entered not only under its original name, but also under the name Staphylococcus asaccharolyticus var. indolicus, reference being made to *Weinberg et al.* (1937). These authors consider Mi. indolicus to be a variety of Staph. asaccharolyticus, deviating from this organism only by a more pronounced production of gas and by forming opaque, lenticular colonies in deep agar.

Hare et al. (1952) and *Thomas & Hare* (1954) referring to the fact that species differentiation of anaerobic cocci is based on certain morphological and biochemical features, and that the presence of fatty acids and sulphur compounds has a marked influence on these features, criticized the use of insufficiently defined media in previous studies and suggested that the number of species could be reduced if well-defined media were employed. Using standardized media these workers were able to arrange the

anaerobic cocci in 9 groups; previously 32 species had been recognized.

In the seventh edition of *Bergey's Manual* (1957), there is no entry of Mi. indolicus, and Staph. asaccharolyticus is included as a synonym to Peptococcus asaccharolyticus. These changes probably reflect the above-mentioned works of *Hare et al.*, which are quoted in the introduction to the description of the genus Peptococcus. Neither is there any mention of Mi. indolicus in the fifth edition of *Topley and Wilson*'s Principles of Bacteriology and Immunity (1964). *Prévot* (1966) and *Prévot et al.* (1967) reaffirm the view that Mi. indolicus is a variant of Staph. asaccharolyticus on the grounds that only insignificant differences in morphological, cultural, and biochemical features exist between the 2 organisms.

The above-mentioned confusion in the taxonomy of anaerobic cocci probably explains why *Fiévez* (1965) has described 15 bovine strains of anaerobic cocci as Peptococcus asaccharolyticus, pointing out that they belong to a group of microorganisms the existence and pathogenicity of which had been studied in particular in material of human origin. It must be assumed, however, that these strains are identical with Mi. indolicus, since they are biochemically very similar to those studied in the present work, and also of similar origin (mammary abscesses in cows).

As regards cell morphology, growth characteristics, and certain biochemical properties, the results of the present work are, on the whole, in agreement with earlier investigations.

Leth Jørgensen (1937) found mucoid and ropy surface colonies on blood agar, whereas *Stuart et al.* (1951) described surface colonies as easily emulsified.

In the present study surface colonies on blood agar were of varying consistence, to some extent seemingly dependent on external factors, i.e., incubation time and humidity of the medium. However, regardless of such factors, some strains would form colonies of a rather constant consistence.

Variations in biochemical characters are described by *Christiansen, Stuart et al., Fiévez,* and *Cornelisse et al.* (1970).

Cornelisse et al. described 23 strains of anaerobic cocci isolated from cases of summermastitis. Strains originating from adult cows were gas-producing and supposedly identical with Mi. indolicus. They were considered to come within the Streptococcaceae and tentatively (*Bergey's Manual* 1957) named Pepto-

streptococcus indolicus. Strains from heifers were non gas-producing and tentatively named Peptostreptococcus uberis.

In the present investigation strains resembling the Peptostreptococcus uberis of *Cornelisse et al.* were not observed; all the strains studied showed an abundant gas-prodution.

Hydrogen sulphide was produced by all strains examined, but the amount produced varied widely depending on the medium employed; this is in accordance with observations in other species of anaerobic cocci (*Hare et al.*). Of the media tested Robertson's cooked-meat medium seemed to be the most suitable for the demonstration of hydrogen sulphide production. In spite of abundant growth, divergent results were obtained when the strains were cultured in ordinary nutrient broth. The 2 media are identical apart from the cooked meat, so presumably the meat is the source of sulphur compounds essential for the production of hydrogen sulphide. The fact that tests for hydrogen sulphide production constantly turned out negative when carried out in ferrous-sulphate agar may perhaps be explained by the inhibitory effect of sodium thiosulphate on the growth of Mi. indolicus.

The medium-dependence of the hydrogen sulphide production may explain why none of the strains described by *Christiansen* were found to produce hydrogen sulphide, while strains described by *Leth Jørgensen* (1937) and later workers were positive in that respect. The medium employed by *Christiansen* being plain nutrient broth, in which production of hydrogen sulphide seems to be inconstant, his strains might very well have proved hydrogen sulphide producing had they been tested under optimal conditions.

In the present investigation all the strains were found catalase-negative, which is in agreement with the findings of *Cornelisse et al.*

The 15 strains described by *Fiévez* all produced coagulase, and in the present investigation coagulase production was demonstrated in 267 of 274 strains.

Fiévez' 15 strains were resistant or moderately sensitive to Penicillin. In the present study a high degree of sensitivity to Penicillin was found in the 20 strains tested. Concerning the sensitivity to other antibiotics, the results obtained agree with those of *Fiévez*. The discrepancy as regards Penicillin sensitivity may indicate that *Fiévez'* strains were not Mi. indolicus. However, his strains reduced nitrate and nitrite, i.e., they conformed in

that respect to the Mi. indolicus strains of this study, but not (*Prévot* 1967) to Staph. asaccharolyticus.

As in the investigations of *Christiansen, Leth Jørgensen* (1937), *Stuart et al.*, and *Fiévez* the tests for fermentation of carbohydrates turned out negative in this study. Furthermore, the citrate utilization test was negative, and urea was not decomposed.

The optimum temperature for growth of Mi. indolicus was determined by *Christiansen* to be 37°—38° C; this result agrees with that of the present study, from which it further appears that it has a low resistance to heat, being killed on momentary heating to 85° C.

As far as the author is aware very little work has been done on the serology of anaerobic cocci, apart from limited studies by means of the agglutination test, and a classification on the basis of antigenic properties has apparently never been attempted.

Christiansen's 2 strains were both specifically agglutinated by an antiserum prepared on rabbits by intravenous injections of one of the strains.

In the present investigation 217 strains of Mi. indolicus were studied by double diffusion-in-gel analysis, whereby these strains could be classified in 6 antigenically different types.

Most likely the specific antigens are polysaccharides, though presumably the crude extracts will have contained a multiplicity of substances; a chemical determination of the antigenic components was not performed.

The Type-B antiserum constantly showed marked cross reactions, the other 5 type sera frequently or occasionally. However, in the precipitin-band patterns reactions of identity could easily be distinguished from reactions of partial identity (Fig. 4).

The antigenic types B, C, D, and E were commonly occurring, with Type C as the one most frequently demonstrated, while only 3 strains could be referred to each of the Types A and F (Table 2).

In each of the 5 areas studied, Types B, C, D, and E were all represented among the strains isolated from cases of summermastitis (Table 3). The 3 strains classified as Type A all originated in the same area, though from different herds.

The antigenic heterogeneity of strains originating from the same herd would seem to suggest that the infections were derived from a variety of sources, but gives no grounds for definite conclusions concerning the epizootiology of summermastitis.

The type distribution of the strains isolated from insects (9 from Hydrotaea irritans var. Fallén and 1 from a Simulium) was similar to that of the strains isolated from cases of summermastitis (Table 4). This, together with the fact that also Corynebacterium pyogenes has been isolated from these species of insects (*Bahr* 1952, 1955, *Overgård Nielsen* 1973, *Høi Sørensen* 1973), supports the assumption that these insects act as vectors of summermastitis.

The strains originating from cases of acute or chronic mastitis in cows or from various suppurative lesions in cattle, as well as those isolated from the vagina or interdigital skin of clinically healthy cows, all fell within the 4 commonest types (Table 4).

Although a very limited number of healthy cattle were examined, the results would seem to indicate that Mi. indolicus may be widely distributed in a herd. A more extensive study of the spread of Mi. indolicus among clinically healthy cattle would be of great interest.

It is interesting to note that 3 of the 9 porcine strains were antigenically different from all the strains of bovine origin.

It should finally be emphasized that, according to *Crowle* (1961), double diffusion-in-gel analysis may sometimes give spurious reactions of identity, as certain antisera may not be capable of distinguishing minor antigenic differences which might be demonstrated by means of other antisera. No such phenomena were experienced in the present study, but under all circumstances it would be more correct if the terms "reaction of identity" and "reaction of partial identity" were replaced by the terms "pattern of fusion" and "pattern of partial intersection", respectively (*Crowle*), as these terms convey exactly what has been observed.

As regards the complement fixation test it was obvious that the differences in titres were due more to properties of the antisera than to differences between the antigens, and that the complement-fixing antibodies were not the same as the precipitating antibodies. The complement fixation test would seem to be useful for differentiating Mi. indolicus from other species of anaerobic cocci (Table 5).

By previous investigators (*Christiansen, Fiévez, Cornelisse et al.*) Mi. indolicus was found to be of little, if any, pathogenicity to small laboratory animals; this is in accordance with the results of the present study.

Christiansen inoculated nutrient broth cultures into the mammary glands of 2 calves and 1 lactating cow, and 1 of the calves developed an acute suppurative mastitis. However, later workers have been unable to reproduce Christiansen's result, be it in heifers, dry cows, or lactating cows (*Stuart et al., Cornelisse et al., Høi Sørensen* 1972 a).

The possible synergism betwcen Mi. indolicus and other organisms (especially Cb. pyogenes) in the causative complex of summermastitis has been discussed by *Leth Jørgensen* (1937, 1966), *Stuart et al.,* and *Høi Sørensen* (1972 a, b).

II. The 4 human strains of anaerobic cocci

These strains were received from the Institut Pasteur, Paris, and classified by that institute as Staphylococcus asaccharolyticus Distaso.

According to *Prévot* (1966), the biochemical criteria for the identification of this organism are: production of indole, hydrogen sulphide, and gas; nitrates not reduced, and carbohydrates not fermented.

None of the 4 strains proved to be in full accordance with these criteria. Thus Strain 2229 reduced nitrate, and Strain 2480, besides reducing nitrate, fermented carbohydrates (Table 1). In Strain 4598 gas production could not be detected, and in Strain 5106 neither indole nor gas production.

According to the original description (*Distaso* 1912) production of gas is very rare in Staph. asaccharolyticus, and, like in other anaerobic cocci (*Hare et al.* 1952) and as confirmed in the present study, greatly dependent on the medium employed. So, probably the failure of Strains 4598 and 5106 to produce gas should be ascribed to properties of the media.

Strain 4598, apart from the lack of gas production, conformed to the description of Staph. asaccharolyticus, biochemically as well as in growth characteristics. Judged by the results of the few biochemical tests performed, and in accordance with the criteria of *Prévot* (1966), Strain 2480 was most in conformity with Staph. aerogenes Schottmüller and Strain 5106 with Staph. anaerobius Jungano, while Strain 2229 was of doubtful identity.

The 4 strains differed markedly from Mi. indolicus in growth characteristics and biochemical properties (Table 1) and showed no serological relationship with it (Table 5).

CONCLUSION

The results of the present study, held together with those of previous investigations, give grounds for formulating the following biochemical criteria for identification of Micrococcus indolicus:

positive	*negative*
indole production	carbohydrate fermentation
hydrogen sulphide production	catalase production
gas production	urea decomposition
coagulase production (variable)	serum-gelatine liquefaction
nitrate and nitrite reduction	citrate utilization

According to the criteria for identification of anaerobic cocci given by *Prévot* (1966) the 4 strains of anaerobic cocci of human origin seem to represent at least 3 species closely related to Mi. indolicus.

The 4 human strains are markedly different from Mi. indolicus in growth characteristics, and do not fulfill the above-mentioned biochemical criteria for identification of Mi. indolicus; furthermore, they show no serological relationship whatsoever to Mi. indolicus. On the other hand, Mi. indolicus, divisible in a number of types by the precipitation test, but possessing a common antigen demonstrable by the complement-fixation test, has proved to be a well-defined species, which should not be classified just as a variant of Staph. asaccharolyticus. Whether it should be classified as Micrococcus or Staphylococcus would appear to be a question of minor importance.

Mi. indolicus seems to be of fairly common occurrence in the skin and mucous membranes of clinically healthy cattle.

The fact that strains of Mi. indolicus isolated from healthy cattle and from specimens of Hydrotaea and Simulium fell within the same serotypes as the vast majority of strains isolated from cases of summermastitis, supports the view that insects are mechanical vectors of that disease, and that, besides clinically infected cattle, healthy cattle may act as sources of the infection.

The finding of 3 porcine strains of a serotype not represented among the strains of bovine origin may be taken as a hint of a possible existence of a certain species-specificity.

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SAMMENDRAG

Micrococcus indolicus. Nogle biokemiske egenskaber samt påvisning af seks antigent forskellige typer.

Undersøgelsen omfatter ialt 274 stammer af Micrococcus indolicus (heraf 211 isoleret fra sommermastitis, 13 fra andre mastiter hos kvæg, 15 fra andre suppurative lidelser hos kvæg, 13 fra insekter, 13 fra vagina eller klovspalte hos klinisk sunde køer og 9 fra forskellige suppurative lidelser hos svin) samt 4 stammer af anaerobe kokker af human oprindelse, som formentlig repræsenterer mindst 3 Mi. indolicus nærtstående arter: Staph. asaccharolyticus Distaso, Staph. aerogenes Schottmüller og Staph. anaerobius Jungano.

Mi. indolicus' kulturelle egenskaber samt de vigtigste biokemiske kriterier for dens identifikation beskrives (tabel 1).

Ved gel diffusions analyse af 217 Mi. indolicus stammer påvistes 6 antigene typer: A, B, C, D, E og F (fig. 2, 3, 4 og 5, tabel 2, 3, 4 og 6), medens der ved komplementbindingsprøve ikke fandtes antigene forskelle mellem de 6 typer (tabel 5).

Stammer isoleret fra klinisk sunde dyr og fra insekter var alle af en af de 4 typer, som hyppigst påvistes blandt stammer isoleret fra sommermastitis.

Tre af 9 porcine stammer tilhørte en type (F), som endnu ikke er påvist hos kvæg.

De 4 humane stammer var antigent forskellige fra Mi. indolicus (tabel 5) og afveg endvidere i biokemiske og kulturelle egenskaber (tabel 1).

Det konkluderes, som formodet af *Christiansen* (1934), at Micrococcus indolicus Christiansen bør klassificeres som en selvstændig species og ikke som en variant af Staphylococcus asaccharolyticus Distaso (*Prévot et al.* 1967).

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