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Characteristics of *Haemophilus ducreyi* in Culture

A. W. STURM[1]* AND H. C. ZANEN[2]

Department of Medical Microbiology, St Laurentius Ziekenhuis, 6040 AX Roermond,[1] and Laboratorium voor de Gezondheidsleer, University of Amsterdam, 1092 AD Amsterdam,[2] The Netherlands

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Growth on different media and the influence of culture conditions were studied on 19 recently isolated strains of *Haemophilus ducreyi*, none of which had more than four passages on artificial media. The results were compared with 10 laboratory strains, which had an unknown number of passages in vitro. For all strains, growth was best on 30% rabbit blood agar and on Bieling agar. The laboratory strains showed a tendency to grow better on chocolate agar than did the fresh isolates. Of 19 fresh clinical isolates, 12 were CO_2 dependent, and 2 needed extra moisture for growth. From the 10 laboratory strains, only one needed CO_2 and none needed extra moisture. All 29 strains grew under anaerobic conditions. Of the 19 fresh clinical isolates, 12 grew at 22°C, but only 2 of the 10 laboratory strains grew at this temperature. The laboratory strains grew better than the fresh isolates at 37°C, and the optimal pH for all strains was pH 6.5 to 7.0. All strains showed starch aggregation.

Bezançon et al. (2) first isolated *Haemophilus ducreyi* on a 30% rabbit blood agar slant from the genital ulcer of a patient with chancroid. Since then there has been controversy over the cultural behavior and colonial appearance of this pathogen, and various media and environmental conditions have been described. A 30% rabbit blood agar (2, 16) and whole blood (4, 17) were the media of choice up to 1978 when Hammond et al. (8) described the isolation of *H. ducreyi* on chocolate agar with IsoVitalex. In 1981, Hafiz et al. (5) first isolated *H. ducreyi* on a medium without blood but containing hemin, corn starch, glucose, glutamine, and cocarboxylase. Later (6), they reported better growth when 0.4% gelatin was added to this medium. In 1981, we described a modification of Bieling medium. This is a medium with lysed horse blood, originally developed for the isolation of gonococci (14). On this medium, we obtained excellent growth with 48 strains of *H. ducreyi* (A. W. Sturm, thesis, University of Amsterdam, 1981). According to Dienst and Augusta (3) and Reyman (13), good growth should be obtained on a suitable medium at 28 to 33°C, but a moist atmosphere is essential. Carbon dioxide did not stimulate growth of the three *H. ducreyi* strains tested by Kilian (10).

The colonial appearance of *H. ducreyi* differs with the medium used. According to Saelhof (15) and Ito (9), pinpoint, grey colonies are seen on rabbit blood agar after 48 h of incubation. If one touches these colonies, they glide over the surface of the medium. In older cultures, colonies become flattened and the color changes from greyish white to yellowish brown (9). Hemolysis on rabbit blood agar is described by Saelhof (15) and Beeson (1), but Stein (16) did not observe hemolysis. Hafiz et al. (6) found a halo of aggregated starch around colonies of *H. ducreyi* grown on their medium.

Because most observations have been made with *H. ducreyi* strains subcultured on artificial media several times, we were interested in the conditions for growth of fresh isolates and how they affect characteristics of *H. ducreyi* grown on various media.

MATERIALS AND METHODS

Bacterial strains. Twenty-nine strains of *H. ducreyi* were used. Nineteen were isolated in Amsterdam from genital ulcers and six came from Winnipeg, Canada. Four were reference strains (ATCC 27721, NCTC 10945, CIP 542, and CIP A76). Our own strains were stored at −70°C, after no more than three passages on rabbit blood agar. When needed, the organisms were subcultured onto the same medium. The other strains were passed every 2 days on rabbit blood agar at least 45 times in our laboratory. We were not informed of the number of passages made in other laboratories. Identification of all strains was done by the methods of Kilian (10).

Growth conditions. Growth was tested on different media: rabbit blood agar (16), chocolate agar with 1% IsoVitalex, supplemented GC agar (7), starch aggregation medium (6) with and without 0.4% gelatin, and modified Bieling agar (Sturm, thesis). Rabbit blood agar was composed of Columbia agar base (Difco Laboratories, Detroit, Mich.) with 30% defibrinated blood and 2.5% yeast dialysate. GC agar (Difco) was supplemented with 1% IsoVitalex and 1% hemoglobin. Starch aggregation medium was composed of GC agar supplemented with 0.01% (wt/vol) hemin, 0.002 g of cocarboxylase per liter and 0.1 g of glutamine per liter. Bieling agar was a mixture of 2 parts Columbia agar base (Difco) and 1 part hemolyzed horse blood, supplemented with 2.5% yeast dialysate. Hemolysis was obtained by heating equal volumes of blood and distilled water at 60°C in a water bath.

Growth was tested on rabbit blood agar under candle jar conditions with and without extra moisture, in a closed jar with moistened air, in a closed anaerobic jar (BBL Microbiology Systems, Cockeysville, Md.) without a catalyst but with two CO_2- and H_2-generating envelopes (BBL no. 70304) (campylobacter conditions), and in an identical jar with catalyst and one CO_2- and H_2-generating envelope (anaerobic conditions).

Growth was tested on rabbit blood agar at 22, 33, 37, and 42°C. Growth was tested on Bieling agar at pH 6, pH 6.5, pH 7, pH 7.5, and pH 8.

With a multipoint inoculator (Denley Instruments Ltd., Billinghurst, Sussex, England), plates were seeded with 0.001 ml of condensation fluid from a 16- to 24-h culture on a rabbit blood agar slant. The plates were incubated for 6 days and were inspected every 48 h.

The appearance of *H. ducreyi* when grown on the different media was studied as described above. Plates were incubated for 48 h in a candle jar and in an anaerobic atmosphere

* Corresponding author.

TABLE 1. Growth of *H. ducreyi* under different conditions

Parameter	% Growth of *H. ducreyi*					
	++[a]		+[a]		−[a]	
	A[b]	B[c]	A[b]	B[c]	A[b]	B[c]
Agar						
30% rabbit blood	84	90	16	10	−	−
Enriched chocolate	−	60	84	40	16	−
Enriched GC	−	40	68	60	32	−
Starch aggregation	−	−	89	100	11	−
Starch aggregation with gelatin	−	−	95	100	5	−
Bieling	89	90	11	10	−	−
Atmosphere						
Candle jar	100	100	−	−	−	−
Candle jar, no H_2O	−	10	89	90	11	−
Candle jar, no CO_2	−	−	37	90	63	10
Anaerobic	−	−	100	100	−	−
Campylobacter conditions	95	90	5	10	−	−
Temperature (°C)						
22	−	−	100	80	−	20
33	100	100	−	−	−	−
37	−	50	100	50	−	−
42	−	−	−	−	100	100
pH						
6.0	−	−	32	30	68	70
6.5	100	100	−	−	−	−
7.0	100	100	−	−	−	−
7.5	−	−	21	80	79	20
8.0	−	−	−	−	100	100

[a] ++, Good growth; +, moderate growth; −, no growth.
[b] Growth of 19 fresh clinical isolates.
[c] Growth of 10 laboratory strains.

(GasPak jar; BBL). Hemolysis was studied on 30% rabbit blood agar and on 30% sheep blood agar plates at 33°C in the candle jar and under anaerobic conditions (GasPak). Starch aggregation was studied as described by Hafiz et al. (6).

RESULTS

The results are compiled in Table 1. Rabbit blood agar and Bieling agar were equal as growth media. Our fresh isolates grew less on enriched chocolate agar and GC agar than did the other strains. All strains grew much slower on starch aggregation medium than on modified Bieling agar, and the addition of gelatin did not alter this.

Campylobacter conditions favored growth of all strains of *H. ducreyi*. Of 19 fresh isolates, 12 did not grow without supplemented carbon dioxide. Moisture was essential for the growth of 2 out of 19 fresh strains. All except one of the laboratory strains grew without CO_2 and none needed extra moisture. All strains grew without oxygen but growth was less luxuriant.

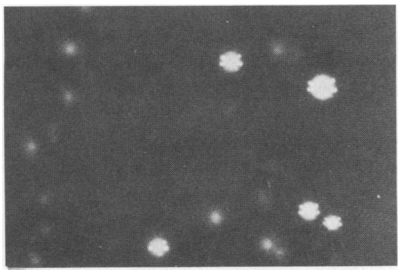

FIG. 1. Polymorphic growth of *H. ducreyi*.

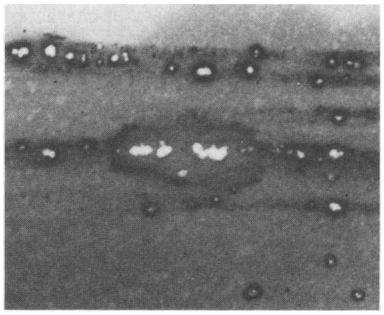

FIG. 2. Starch aggregation of *H. ducreyi*.

All strains grew best at 33°C after overnight incubation. Visible growth was seen at this temperature with 6 of the 19 fresh strains and with 9 of the 10 laboratory strains. All strains grew well after 48 h. All 19 fresh isolates grew at 22°C. After an incubation period of 2 days, 12 showed moderate growth. The other 7 showed visible growth after 4 to 5 days. Of the laboratory strains, only two grew after 2 days, six grew after 4 to 5 days, and two did not grow at all.

The optimal pH value was pH 6.5 to pH 7.0 for all strains of *H. ducreyi*.

After an incubation period of 48 h on a rabbit blood agar plate at 33°C in a candle jar, an area of heavy growth showed colonies that were pyrimidal in shape and had a smooth, glistening surface. They had an average diameter of ±0.5 mm. If growth was less heavy, the colonies had a diameter of ±1.0 to 1.5 mm and a flat granular surface. This type of colony had a brown-yellow color. Between heavy and less-heavy growth, both colony types were present, giving a polymorphic appearance. This polymorphic growth (Fig. 1) was also seen on Bieling agar but not on media incubated without oxygen. There was a uniform colonial appearance, as in the area with heavy growth. Colonies of both types glided easily over the surface of the medium and were autoagglutinable.

Hemolysis was seen on rabbit blood agar with two fresh strains. On sheep blood agar, 21 strains (16 of which were fresh isolates) showed α-hemolysis, and anaerobiosis did not change this.

Starch aggregation was found with all strains (Fig. 2).

DISCUSSION

Fresh clinical isolates of *H. ducreyi* differ from older strains in essential points (Table 2). This has implications for the isolation of *H. ducreyi* from clinical material.

Under candle jar conditions, the best growth of *H. ducreyi* is obtained on 30% rabbit blood agar and on Bieling agar. In most laboratories, it is easier to prepare this latter medium, because there is no need for large volumes of sterile, defibrinated rabbit blood. In our hands, Bieling medium gives better results than enriched chocolate agar, as recommended by Hammond et al. (8). On the starch aggregation medium described by Kinghorn et al. (11), *H. ducreyi* grows with tiny colonies even after 4 days of incubation, and we have not found better growth by adding gelatin. However, the aggregation of starch around the colony makes it easier to recognize colonies of *H. ducreyi*. Supplementary CO_2 has been found essential for growth of 12 of 19 fresh clinical isolates. Kilian (10) did not find CO_2 dependence, but he worked only with laboratory strains. Moisture stimulates the growth of most *H. ducreyi* strains, but it has been found to be essential for only 2 out of 29. Anaerobic growth was obtained with all 29 strains. This is not in accordance with

TABLE 2. Summary of characteristics of fresh isolates and laboratory strains of *H. ducreyi* in culture

H. ducreyi (no.)	Characteristics in culture (% total)							
	CO_2 dependence	H_2O dependence	Moderate growth at 22°C	Growth[a] at 37°C		Growth[a] on chocolate agar		
				+	++	−	+	++
Fresh isolates (19)	12 (63)	2 (11)	12 (63)	19 (100)		3 (16)	16 (84)	0
Laboratory strains (10)	1 (10)	0	2 (20)	5 (50)	5 (50)	0	4 (40)	6 (60)

[a] −, No growth; +, moderate growth; ++, good growth.

the observation of Oberhofer and Back (12) who described *H. ducreyi* strains as obligatory aerobes. All our strains have grown best under campylobacter conditions. To our knowledge, this is the first time that this has been reported.

The optimum temperature for clinical isolates (33°C) is compatible with other reports (3, 13), which state that this is between 28 and 33°C. So the isolation of *H. ducreyi* from clinical material should be done at a temperature of ±30°C. There is a tendency to grow at temperatures below that of the human body, but growth at 22°C has never been reported before. It thus appears that *H. ducreyi* differs from many of the causative agents of venereal diseases, which fail to survive at lower temperatures.

H. ducreyi does not grow in alkaline media, and on acid media the growth is less than on media with a neutral pH.

The colonial appearance of *H. ducreyi* is as described previously (9, 16). If there is a heavy growth, colonies can be similar to those of streptococci. When growth is less luxuriant they look like those of diphtheroids. Hemolysin production is a variable characteristic.

In our laboratory, the isolation of *H. ducreyi* from clinical specimens was performed by the use of modified Bieling agar as plates and as slants (Sturm, thesis). One of each is incubated in a candle jar at 33°C. To these penicillin (0.1 IU/ml) and polymyxin (7.5 mg/liter) are added. The other two are incubated anaerobically. To these metronidazole (5 mg/liter) is added.

Campylobacter conditions favor growth of so many bacterial species that the advantage of better growth is overruled by the disadvantage of competition with other bacterial species. If a good selective medium could be developed, we think that campylobacter conditions would be the gas mixture of choice.

Knowledge of the colonial appearance of fresh clinical isolates of *H. ducreyi* is as important for the bacteriological diagnosis of chancroid as are the use of a suitable medium and optimal growth conditions. Starch aggregation could be helpful if the medium on which this characteristic can be recognized was able to give better growth.

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