

Characteristics of *Haemophilus ducreyi*

A study

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SUMMARY In a study of 13 local and four reference strains of *Haemophilus ducreyi* all grew well on a selective medium consisting of Bacto proteose No 3 agar (Difco), soluble starch, IsoVitalax, human blood, and vancomycin. All the strains reduced nitrate, were alkaline-phosphatase-positive, and (with one exception) used glucose, fructose, and mannose. β -lactamase was produced by 12 local strains. Erythromycin was the most effective antibiotic tested, followed by streptomycin, co-trimoxazole, and spectinomycin.

Introduction

Chancroid has been traditionally regarded as a tropical disease which rarely occurs in temperate countries. In recent years, however, there have been reports of its incidence in Greenland,¹ Canada,² the United States,^{3,4} and Britain.⁵ This is largely due to the introduction of a selective medium for the isolation of *Haemophilus ducreyi*.⁶

Despite the greater interest being shown in the disease, some of the characteristics of the organism are still not established. In this paper we report the characterisation of 13 local strains, which were isolated in 1979. A reference strain (CIP 54.2) from the Institut Pasteur, Paris, and three other strains (C1, C2, and C3) (obtained by courtesy of W L Albritton, Canada) were included in the study. Our findings suggest that *H ducreyi* is able to utilise carbohydrates and reduce nitrate. The oxidase reaction was weak, but this could have been due to the non-specific action of haemin.

Materials and methods

CULTURE MEDIA

The medium used to grow the organism was made up of Bacto Proteose No 3 agar (Difco), to which was added soluble starch 0.1%, IsoVitalax 1%, human blood 15%, and vancomycin 3 μ g/ml. Plates of medium were sealed in a plastic bag and kept at 4°C until use within a week. Cottonwool swabs saturated with normal saline were used to collect culture

samples from the penile ulcers of patients presenting with clinically diagnosed chancroid. Specimens were inoculated directly on to the plates of medium, which were then kept in a candle-extinction tin together with a piece of moist cottonwool. The plates were incubated at 35°C and read after 48 hours and daily thereafter. The strains were identified presumptively by their colonial morphology and by microscopy. The liquid medium which was used to grow the organism consisted of Bacto Proteose Peptone No 3 (Difco) 1%, sodium chloride 0.5% and fetal bovine serum 10%.

BIOCHEMICAL TESTS

Tests for oxidase,⁷ alkaline phosphatase,⁸ catalase, the ability to biosynthesise porphyrin from δ -amino-laevulinic acid (ALA),⁹ and β -lactamase¹⁰ have been described. To improve the oxidase reaction 24-hour cultures of the organisms were suspended in phosphate-buffered saline pH 7.2 and centrifuged.¹¹ The supernatant fluid was removed and the sediments deposited on a piece of filter paper. Oxidase reagent was then dropped on to the sediments and the changes observed. The activity of haemin on the oxidase reagent, NNN'N'-tetramethyl-p-phenylenediamine dihydrochloride, was determined by preparing a 20% solution of haemin (Sigma H-2375 equine type III) in 1 mol/l sodium hydroxide and diluting it in distilled water to give a final concentration of 800 μ g/ml. This dilution was dropped on to one end of a strip of filter paper, and to the other end was added a drop of oxidase reagent. The development of a purple colour was looked for at the interface between the two reagents.

The requirement for V factor was tested by the ability of the organisms to grow on a medium made up of GC medium base (Difco), haemoglobin 1%,

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glucose 0.1%, and L-glutamine 0.01%. Nitrate reduction was tested by suspending a heavy inoculum of the organism in a liquid medium consisting of Bacto Proteose Peptone No 3 (Difco) 1%, sodium chloride 0.5%, soluble starch 0.1%, and potassium nitrate 0.1%. After six hours' incubation at 35°C, reagents A and B were added as described.¹²

Carbohydrate utilisation was tested by a reagent-impregnated filter paper method (unpublished data). Briefly, the reagents consisted of Bacto Proteose Peptone No 3 (Difco) 1%, L-cysteine 0.026%, carbohydrate 5%, and bromocresol purple 0.2% dissolved in 0.025 mol/l Sorensen's phosphate buffer pH 7.2. Pieces of Whatman's No 1 filter paper, measuring 4 × 1 cm were placed on Petri dishes and saturated with the respective reagents. Heavy inocula of 24-hour organisms were then smeared on the surface of the paper strips. Pieces of wet cottonwool were also deposited on the Petri dishes, which were then placed in a tin containing some water. The lid was closed and the tin incubated at 35°C for two hours. Carbohydrate utilisation was indicated by the presence of yellow zones around the inocula. The paper remained purple if there was no utilisation. The carbohydrates tested were arabinose, xylose, glucose, fructose, mannose, galactose, lactose, maltose, and sucrose.

Other biochemical reactions were also tested using the MICRO-ID (General Diagnostics) system for identifying members of the *Enterobacteriaceae*. The system tested for Voges-Proskauer reaction, nitrate reduction, phenylalanine deaminase, hydrogen sulphide, indole, ornithine decarboxylase, lysine decarboxylase, malonate utilisation, urease activity, esculin hydrolysis, β -galactosidase, and fermentation of arabinose, adonitol, inositol, and sorbitol. The manufacturer's directions were followed except in the inoculation of the organism. The clear plastic tape covering the test wells was cut away and the organism directly inoculated on to the test discs; 0.2 ml normal saline was then dropped on to each disc and a transparent sealing tape used to cover the test wells.

SENSITIVITY TESTS

The minimum inhibitory concentrations (MICs) of ampicillin, erythromycin, tetracycline, streptomycin, sulphadiazine, co-trimoxazole (trimethoprim: sulphamethoxazole ratio, 1 : 19), spectinomycin, vancomycin, colistin, and amphotericin B were determined by the agar dilution technique of Hammond *et al*¹⁴ using GC medium base (Difco), haemoglobin 1%, IsoVitalax 1% (except for sulphadiazine and co-trimoxazole where Enrichment 4¹⁵ and glucose 0.1% were used) and doubling dilutions of antimicrobial agents.

Results

Good growth of all the strains was obtained with the medium described. In the early phase of our study various types of media were used, some of which were too inhibitory. Different types of agar had varying effects on *H ducreyi*. Thus, better growth was obtained using Bacto purified (Difco) or Baltimore Biological Laboratory (BBL) purified agar than BBL granulated or Oxoid agar No 1. In the formulation chosen soluble starch was unnecessary for all the strains except CIP 54.2, which grew better with it.

COLONIAL GROWTH

Most of the strains produced colonies of 0.5-1 mm in diameter in 24 hours and of 1-2 mm in 48 hours. They were whitish in colour but might have a pale yellow-brown hue in the heaped up areas where the growth was confluent. They could be pushed intact across the agar surface. It was difficult to make a uniform suspension of the colonies in a drop of water as the organisms were stuck together in clumps. In broth culture granular growth was seen after 24-48 hours. The clumps tended to adhere to the side of the testtube. After 3-5 days long filaments were seen, giving a woolly appearance.

MICROSCOPICAL EXAMINATION

Microscopical examination of Gram-stained colonies from solid medium showed that few of the organisms occurred as isolated Gram-negative bacilli. Most of them were present as members of microcolonies. When 24-hour cultures were examined, the organisms at the edge of some of the microcolonies tended to be arranged in parallel rows, giving a wavy appearance. Long chains of organisms were more evident when the cultures were taken from liquid medium. These were sometimes arranged in loops, and occasionally parallel rows produced the classical "railway track" appearance.

BIOCHEMICAL REACTIONS

All the strains grew in the absence of V factor and were unable to biosynthesise porphyrin from ALA. They were all catalase-negative but positive for alkaline phosphatase and nitrate reduction. The oxidase reaction was slow to develop with the traditional method.⁷ After 10-15 minutes a pale purple colour appeared on and around the inocula; the colour darkened with time. When the cells were first washed in phosphate-buffered saline, the purple colour developed within 3-4 minutes of the reagent being added. On the other hand, haemin itself was also able to turn the oxidase reagent purple within a few minutes. When a small piece of uninoculated

medium was added to the filter paper, a similar darkening of the agar occurred on the undersurface.

Sixteen of the 17 strains were able to utilise glucose, fructose, and mannose by the filter paper method. The reference strain CIP 54.2 was unable to utilise any of the carbohydrates. For consistent results heavy inocula from 24-hour cultures were used. A high humidity was necessary to reduce evaporation to a minimum. There was no difficulty in distinguishing a positive yellow zone from a pallor around some of the inocula due to excess moisture. All the strains gave similar results for the MICRO-ID system. Of the 15 tests, only nitrate reduction gave a positive result.

β -lactamase was produced by 12 local strains and none of the reference strains (table). All the β -lactamase producers had MICs for ampicillin >8 $\mu\text{g/ml}$. Erythromycin was the most effective antibiotic, followed by streptomycin, co-trimoxazole, and spectinomycin. A high percentage of the strains was resistant to tetracycline, although all were uniformly resistant to vancomycin and amphotericin B.

Discussion

The isolation of 13 strains of *H ducreyi* from genital ulcers confirms the value of a selective solid medium for diagnosing chancroid. The organism is fastidious in its growth requirements. The optimum medium should, therefore, be able to provide adequate hydration, necessary enrichment, and minimal toxicity. The addition of whole blood or serum to the basal medium contributes greatly to providing these conditions. Fetal bovine serum is able to support

good growth of *H ducreyi*,¹⁶ but in our laboratory we have been using human blood as it is cheaper and readily available from the blood bank.

The characteristic features of *H ducreyi* on solid medium are usually adequate for a presumptive diagnosis. On microscopical examination of young cultures the organism may have wavy parallel rows at the edge of microcolonies. Smears from broth cultures show long chains of cells, which may be arranged in the form of loops. If these features are not seen, microscopical examination may still be useful in excluding the possibility of contamination. Thus, if a uniform suspension is obtained, and most of the cells are isolated Gram-negative bacilli, the organism may not be *H ducreyi* or it may be a mixed culture.

Some uncertainty about the oxidase reactivity of the organism exists. While a negative reaction has been reported by some,^{5,6,9} others have reported a positive reaction.^{3,16} We found that haemin itself can cause a non-specific reaction and the medium can darken even in the presence of oxidase reagent. If the colonies are too acid the oxidase reaction may be negative, especially with organisms which are weakly positive.¹¹ By washing the cells first in phosphate-buffered saline pH 7.2, the oxidase reaction was stronger. Nevertheless, further work is necessary to clarify the extent to which haemin may contribute to the reaction. Perhaps the benzidine method of Faller and Schleifer¹⁷ can be used to determine this.

The organism is able to reduce nitrate, though some strains^{6,16} have yielded negative results. Initially our strains were incubated for several days so that growth could occur before the test reagents were added. Unpredictable results were obtained with

TABLE Susceptibility of *H ducreyi* to antimicrobial agents

Strains	β -lactamase-production	Minimum inhibitory concentrations ($\mu\text{g/ml}$)									
		AMP	ERY	TET	STR	SLD	SXT	SPE	VAN	COL	AMP B
1	+	>8	≤ 0.5	4	2	16	2	16	>16	4	8
4	+	>8	4	8	4	16	2	4	>16	4	>8
5	+	>8	≤ 0.5	8	2	8	4	8	>16	8	>8
6	+	>8	1	8	2	8	4	8	16	16	>8
7	+	>8	4	>8	4	>16	8	16	>16	4	>8
9	+	>8	1	4	4	>16	8	16	>16	2	>8
11	+	>8	≤ 0.5	4	4	>16	4	4	>16	2	>8
12	+	>8	2	>8	4	>16	8	8	>16	4	>8
25	+	>8	≤ 0.5	>8	16	>16	4	8	>16	4	>8
27	+	>8	≤ 0.5	>8	4	>16	2	8	>16	16	>8
30	-	0.5	1	>8	8	16	2	8	>16	16	>8
31	+	>8	≤ 0.5	>8	8	>16	4	4	>16	16	>8
32	+	>8	≤ 0.5	8	8	>16	4	8	>16	8	>8
54.2	-	0.5	≤ 0.5	1	2	4	2	8	16	8	>8
C1	-	≤ 0.25	≤ 0.5	0.5	8	8	4	16	16	8	>8
C2	-	≤ 0.25	≤ 0.5	1	4	4	2	16	>16	4	>8
C3	-	≤ 0.25	≤ 0.5	0.5	4	4	2	8	>16	4	>8

+ = Positive, - = negative

AMP = Ampicillin, ERY = erythromycin, TET = tetracycline, STR = streptomycin, SLD = sulphadiazine, SXT = co-trimoxazole, SPE = spectinomycin, VAN = vancomycin, COL = colistin, AMP B = amphotericin B

certain strains. We later found that by reducing the incubation period to six hours all the strains were consistently positive. This was also confirmed by the positive results which were obtained for nitrate reduction using the commercial MICRO-ID system.

Test results for carbohydrate utilisation have been negative^{4,9} or equivocal.^{16,18} In this study the three hexose sugars—glucose, fructose, and mannose—were consistently utilised by all the strains except CIP 54.2. Compared with the liquid medium systems the filter paper method had better ratios of cells to reagents, and this probably accounted for the more consistent results. Prolonged storage and repeated subculturing may have caused strain CIP 54.2 to lose the ability to utilise the sugars.

The high percentage of strains producing β -lactamase indicates that the use of ampicillin is pointless in Singapore. The enzyme is mediated by a 6.0 megadalton plasmid,¹⁹ which is similar to the TEM-1-type.²⁰ Some recent evidence, however, suggests that more than one plasmid may be involved.³ A comparison of MICs of antibiotics for strains from different regions is limited by the difficulty of standardising methodology. Inoculum size and reading criteria will need to be uniform. Our in-vitro results, however, corroborate local clinical experience with various antimicrobial agents; poor results have been obtained with both sulphonamide and tetracycline and good results with streptomycin and cotrimoxazole.^{21,22} The value of erythromycin as a therapeutic agent should be explored further in view of its efficacy in vitro and good clinical results.⁴

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