J Clin Pathol 1987;**40**:1174–1177

Selenium and the growth of *Haemophilus ducreyi*

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SUMMARY One of the growth media in current use for *Haemophilus ducreyi* comprises Mueller Hinton agar, chocolatised horse blood, serum and IsoVitalex (BBL). For a better understanding of growth factors, attempts were made to simplify this complex medium. The horse blood was replaced by haemin (200 μg/ml), the serum by albumin (0·2%), and IsoVitalex was substituted only by L-glutamine 0·01%. Most of the strains grew, but when selenium ions were added in a concentration of $3{\cdot}25 \times 10^{-3}$ μg/ml, growth was stimulated and became more luxuriant than growth on conventional media.

In spite of the development of new media[1–3] the isolation rate for *Haemophilus ducreyi* is still low. As it is not known what growth factors other than haemin are required, the composition of growth and isolation media is usually very rich and complex. Normally, only vancomycin is added to isolation plates to inhibit Gram positive organisms.

In a previous study[4] a medium consisting of Mueller Hinton agar, haemin 200 μg/ml, glucose 0·1%, L-glutamine 0·01%, cysteine hydrochloric acid 0·05% and albumin 0·2% was used for antimicrobial susceptibility testing. A low and narrow minimum inhibitory concentration range for sodium selenite (1–4 μg/ml) and copper II chloride (2–8 μg/ml) was found. In this study we tried to simplify further this medium and to examine the role of selenium and copper ions.

Material and methods

Isolates from South Africa (RC Ballard), Kenya (H Nsanze), Thailand (P Echeverria) and the Pasteur Institute Paris (542, 7866, and 76118) were included.

Mueller Hinton agar (BBL Microbiology Systems) supplemented with haemin 200 μg/ml (BDH) was used as a base in each test. This base was sterilised by heat as indicated by the manufacturer (121°C, 15 minutes). Stock solutions of the test products were sterilised by filtration and added to the base to give the desired final concentrations. These products were: bovine albumin fraction V (Sigma), L-glutamine (Merck), cysteine hydrochloride (BDH), glucose (Merck), glutamate (Calbiochem), glutathione (Boehringer, reduced), sodium selenite ($Na_2SeO_3.5H_2O$, Riedel-de Haen), copper chloride ($CuCl_2.2H_2O$, Merck).

Two different media were used as controls. One consisted of Mueller Hinton agar, chocolatised horse blood 5% (Gibco), calf serum 5% (Gibco), and IsoVitalex 1% (BBL). The other medium had the same composition, except for the horse blood which was substituted by haemin 200 μg/ml.

Plates were streaked with a loopful of organisms and incubated for 48 hours in a microaerophilic and humid atmosphere at 33°C. Strains that showed growth were subcultured on the same medium and incubated in similar conditions.

Results

Strains were given a + score when growth after subculture was comparable with growth on the control plates. If growth was more luxuriant than the control, ++ was given. A hazy growth was indicated as ±.

As glucose, L-glutamine, and cysteine hydrochloric acid are generally considered to be the three components of IsoVitalex that are really necessary for *H ducreyi*, these products were first investigated on a limited number of strains (table 1). All strains grew well in the presence of L-glutamine, alone or in combination with the other compounds. Cysteine hydrochloric acid and glucose were not able to support or enhance growth. Growth was diminished in the absence of albumin.

The question arose whether *H ducreyi* specifically requires L-glutamine for growth, or whether glutamate as a precursor, or glutathione as a generated product from glutamine would have the same effect.

Accepted for publication 16 April 1986

Table 1 *Influence of glucose, L-glutamine, and cysteine-hydrochloric acid with and without albumin on the growth of H ducreyi*

Compounds added to the base[1]	*Strain (n = 8)*			
	++[2]	+[3]	±[4]	−[5]
Albumin 0·2%, L-glutamine 0·01%	0	8	0	0
Albumin 0·2%, cysteine-hydrochloric acid 0·05%	0	0	0	8
Albumin 0·2%, L-glutamine 0·01%, cysteine-hydrochloric acid 0·05%	0	8	0	0
Albumin 0·2%, L-glutamine 0·01%, cysteine-hydrochloric acid 0·05%, glucose 0·1%	0	8	0	0
L-glutamine 0·01%	0	0	8	0
Cysteine-hydrochloric acid 0·05%	0	0	0	8
L-glutamine 0·01%, cysteine-hydrochloric acid 0·05%	0	0	8	0
L-glutamine 0·01%, cysteine-hydrochloric acid 0·05%, glucose 0·1%	0	0	8	0
Control plates	0	8	0	0

[1]base: Mueller Hinton agar + haemin (200 μg/ml).
[2]++: Luxuriant growth.
[3]+ : Good growth (comparable with control plates).
[4]± : Hazy growth.
[5]− : No growth.

Table 2 gives the results. Only L-glutamine was able to make the strains grow. Good growth (+) was seen at a concentration of 0·01%. Two strains failed to grow at this concentration, yet they showed a hazy growth at higher concentrations.

Table 3 shows the influence of sodium selenite. Sodium selenite alone, in concentrations 10, 100, and 1000 times less than the minimum inhibitory concentration value, has no effect on the strains, but combined with L-glutamine, a very good growth that was at least comparable (+) and in most cases more luxuriant (++) than that on the control plates was seen.

Table 4 gives the results with copper II chloride. Copper II chloride alone does not support growth, but combined with L-glutamine is comparable with the results obtained with L-glutamine and albumin as the only additive.

Discussion

H ducreyi has always been known to be a fastidious organism. Growth conditions are generally accepted as 33–35°C and a humid atmosphere with reduced oxygen and enhanced carbon dioxide tension. Growth factors, however, are still poorly understood. As a consequence isolation media are very rich and hardly selective so that isolation ratios have always been low.

In an attempt to investigate *H ducreyi* growth factors we tried to simplify one of the conventional media, composed of Mueller Hinton agar, chocolatised horse blood 5%, serum 5%, and IsoVitalex 1 or 2% (BBL). The horse blood was replaced by haemin 200 μg/ml and the serum by albumin 0·2%. Growth in the presence of albumin was comparable with control growth, but without albumin, growth was much less. *H ducreyi* prefers a high protein concentration, rather than some specific serum factor. This is not surprising as carbohydrate metabolism cannot be detected by conventional sugar fermentation tests, while aminopeptidases and proteases are common.[5]

The only component from IsoVitalex really required by *H ducreyi* is L-glutamine (table 1) in a concentration of 0·01%. This is exactly the concentration of glutamine in a medium supplemented with 1% IsoVitalex. It is likely that the strains we used were adapted to this concentration by isolation and subculturing on the classical medium. Some strains still failed to grow.

Table 2 *Influence of L-glutamine, glutamate, and glutathione in various concentrations on the growth of H ducreyi (strains = 12)*

	Base[1] *+ albumin 0·2%*											
	L-glutamine				*Glutamate*				*Glutathione*			
C (% w/vol)	++[2]	+[3]	±[4]	−[5]	++	+	±	−	++	+	±	−
0·1	0	0	0	12	0	0	0	12	0	0	0	12
0·05	0	0	12	0	0	0	0	12	0	0	0	12
0·025	0	0	12	0	0	0	0	12	0	0	0	12
0·01	0	10	0	2	0	0	0	12	0	0	0	12
0·005	0	0	10	2	0	0	0	12	0	0	0	12

Control plates for all 12 strains: +.
[1]base: Mueller Hinton agar + haemin (200 μg/ml).
[2]++: Luxuriant growth.
[3]+ : Good growth (comparable with control plates).
[4]± : Hazy growth.
[5]− : No growth.

Table 3 *Influence of sodium selenite alone and in combination with L-glutamine on the growth of H ducreyi*

Sodium selenite concentration (μg/ml)	*Base*[1] *+ albumin 0·2%*							
	Without L-glutamine (strains n = 9)				*With L-glutamine (strains n = 19)*			
	++[2]	+[3]	±[4]	−[5]	++	+	±	−
0·1	0	0	0	9	0	7	5	7
0·05	0	0	0	9	0	14	0	5
0·01	0	0	0	9	19	0	0	0
0·005	0	0	0	9	0	0	19	0
0·001	0	0	0	9	0	0	19	0
Control plates	0	9	0	0	0	19	0	0

[1]base: Mueller Hinton agar + haemin (200 μg/ml).
[2]++: Luxuriant growth.
[3]+ : Good growth (comparable with control plates).
[4]± : Hazy growth.
[5]− : No growth.

Table 4 *Influence of copper II chloride alone and in combination with L-glutamine on the growth of H ducreyi*

Copper II chloride concentration (μg/ml)	*Base*[1] *+ albumin 0·2%*							
	Without L-glutamine (strains n = 19)				*With L-glutamine (strains n = 19)*			
	++[2]	+[3]	±[4]	−[5]	++	+	±	−
0·1	0	0	0	19	0	12	7	0
0·05	0	0	0	19	0	17	2	0
0·01	0	0	0	19	0	9	10	0
0·005	0	0	0	19	0	16	0	3
0·001	0	0	0	19	0	15	1	3
Control plates	0	19	0	0	0	19	0	0

[1]base: Mueller Hinton agar + haemin (200 μg/ml).
[2]++: Luxuriant growth.
[3]+ : Good growth (comparable with control plates).
[4]± : Hazy growth.
[5]− : No growth.

Surprisingly neither glutamate nor glutathione are able to support growth (table 2).

In view of previous studies of minimum inhibitory concentration[4] we examined the influence of selenium ions in concentrations below the minimum inhibitory concentration. Selenium ions alone were not able to support growth. In combination with glutamine, however, all strains showed luxuriant growth (table 3). The optimal sodium selenite concentration was found to be 0·01 μg/ml, corresponding to $3·25 \times 10^{-3}$ μg/ml selenium ions.

Glutathione is the substrate of the enzyme glutathione peroxidase, which is a seleno enzyme. The combination of glutathione and selenium ions was not able to support growth. It seems, therefore, that glutamine and selenium act separately. The action of selenium is a very specific one, as copper ions do not have any influence, in spite of almost comparable minimum inhibitory concentration values (table 4) (1–4 μg/ml for sodium selenite, 2–8 μg/ml for copper II chloride).

As the optimal selenium concentration is so critical some interesting consequences can be considered. Selenium is a trace element spread across the world with a considerable variety of forms. The selenium concentrations in blood and body fluids reflect dietary intake, which in its turn reflects environmental selenium.[6–8] Thus it would be worthwhile to examine the role of selenium in the pathogenesis of chancroid.

On the other hand, most isolation media are supplemented with whole blood and serum. As selenium concentration is crucial, batches of blood and serum, differing in selenium concentration, should give different isolation ratios. In regions where soil and water selenium concentration are high, one can also expect an influence from the water used to prepare media, if this is not properly deionised.

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