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Biochemical and serological characterization of mycoplasma strains isolated from the genital tracts of humans in Nigeria

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Summary

Fifty-five (55) Mycoplasma strains isolated from the genital tracts of humans were biochemically characterized using various biochemical tests and also serologically identified by growth inhibition technique using 5 mycoplasma antisera namely *M. hominis* PG21; *M. genitalium* G37; *M. penetrans* GTU54 and 2 strains of *M. fermentans* PG18 (HRC 6-62-S-170 and M B713-501-069). Biochemically, 43 (78.2%) strains were identified as *Mycoplasma hominis*, 8 (14.5%) strains as *M. fermentans* and 4 (7.3%) as *M. penetrans*. The *M. hominis* strains hydrolyzed only arginine while the *M. fermentans* and *M. penetrans* strains in addition to arginine hydrolysis also broke down glucose fermentatively and oxidatively. The *M. fermentans* strains showed varying reactions to phosphatase activity and to the reduction of tetrazolium chloride. Serologically, 4 (7.3%) mycoplasma strains were confirmed as *M. penetrans* GTU54 and of the 8 *M. fermentans* strains, 4 (7.3%) were identified as *M. fermentans* PG18 serotype HRC 6-62-S-170 and the other 4 (7.3%) as *M. fermentans* PG18 serotype MB 713-501-069. Only 13 (30.2%) of the 43 *M.hominis* strains were identified as *M. hominis* serotype PG21. None was identified as *M.genitalium*. The heterogeneity of the mycoplasma strains especially *M. hominis* was observed in this study and the need for the use of multiple antisera in growth inhibition test is hereby supported.

Keywords: *Human genital mycoplasmas; biochemical tests; mycoplasma antisera.*

Résumé

Cinquante cinq sous type de mycoplasmes ont été isolés des voies génitales des humains et biochimicallement caractérisé par des tests biomédicaux et identifié par la technique d'inhibition en culture séroligiquement utilisant des antisera. Biochimiquement 43(78.2%) étaient identifié comme mycoplasmes *Hominis*, 8(14.5%) *M. fermentans* et 4 (7.3%) *M. penetrans*. Les *M. Hominis* s'hydrolysait seulement a l'arginine a lorsque *M. fermentans* et *M. penetrans* hydrolisaient a l'arginine et se décomposaient en glucose par fermentation et oxidation. Les *M. fermentans* montraient différentes réactions aux activités des phosphatase et a la réduction au tetrazolium de chloride. Sérologicalement 4 (7.3%) des mycoplasmes sous type étaient confirmes comme *M. penetrans* GTU54 et sur les 8 *M. fermentans*, 4(7.3%) étaient identifié comme *M. fermentans* PG18 sérotype HRC6-62-S-170 et l'autre 4(7.3%) identifié comme *M. fermentans* PG18 serotype MB 713-501-069. Seulement 13(30.2%) des 43 *M.Hominis* étaient identifié comme *M. Hominis* serotype PG21. Aucun n'était identifié comme *M genitalium*. L'héterogénecite des sous type de mycoplasmes spécialement *M.Hominis* était observé dans cette étude et le besoin de l'usage de multiples antisera dans l'inhibition des tests de culture est ainsi supporté.

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Introduction

Mycoplasmas are the smallest bacteria capable of independent existence. The first account of the isolation of a mycoplasma of human origin was from a Bartholin's gland abscess [1]. Since then mycoplasmas have been found to be common inhabitants of the oropharygeal, genital mucous membranes and occasionally other sites as well. In early studies of human mycoplasma, they were not speciated but were assigned to this group of organisms almost exclusively on the basis of cultural characteristics. However, Nicol and Edward [2] used cultural, morphologic, biochemical and serologic properties to achieve the differentiation between groups of human genital and oral mycoplasma. A similar study was carried out by Freundt [3] thus leading to the formal proposal for the establishment of the 3 species of mycoplasmas first associated with humans [4,5]. These species are: *M. hominis*, *M. fermentans* and *M.salivarum*. Aluotto *et al* [6] worked with over 22 species of mycoplasma and were able to modify and standardize biological procedures used to characterize and differentiate the mycoplasmas.

The roles of genital mycoplasmas (Mycoplasma spp. and *Ureaplasma urealyticum*) as disease causing agents have been reported in many parts of the world and Africa [7-9]. Studies on the isolation of mycoplasmas from the genital tracts of adult Nigerians have earlier been reported [10,11]; however, the emergence of new species of genital mycoplasmas with possible pathogenic roles has since been reported from other countries [12-14]. This investigation was therefore undertaken to characterize and identify 55 (fifty-five) mycoplasma strains isolated from

the genital tracts of adult Nigerians using biochemical and serological studies in order to establish the possible presence of emergent species probably associated with genital tract infections.

Materials and methods

Organisms: These are 55 mycoplasma strains isolated from the genital tracts of adult Nigerians attending the Antenatal and Gynaecology Clinics of the University College Hospital, Ibadan between September 2002 and March 2003. The organisms, already cloned for purity, were subcultured into fresh mycoplasma broth [15] and incubated for 48 hours before being used as the inocula for both the biochemical and serological tests. The isolation methods used were as described by Okwoli *et al* [16] whereby the specimens upon collection were first inoculated into mycoplasma broth [15] and after incubation in air at 37°C for 3 days were subcultured onto corresponding mycoplasma agar. The agar plates were incubated under increased carbon dioxide atmosphere in a candle jar at 37°C. They were examined every two days for up to 10 days for the presence of typical mycoplasma colonies.

Digitonin sensitivity [17]: This was used to test the ability of the strains to utilize sterol and the disc growth inhibition method was used [18]. It was performed by first incorporating 0.02ml of 1.5% (w/v) ethanolic solution of digitonin (E Merck, Darmstadt, Germany) in sterile filter paper discs (6.0mm in diameter) and allowed to dry at 37°C. Using the running drop technique [19], a drop of each mycoplasma culture was inoculated on well-dried mycoplasma agar plates and a digitonin disc placed on the centre of each streak. The plates were incubated at 37°C in a candle jar for up to 3 days before being examined for the presence/absence of zones of inhibition around the discs.

Biochemical tests: The basal medium used for most of the tests is the modification of Aluotto *et al* [6] and comprises as follows: Brain heart infusion (BHI) broth, 36.4ml; horse serum, 5.0ml; 10% yeast extract, 2.5ml; 0.2% DNA solution, 0.5ml and 20,000 IU Penicillin solution, 0.1ml. Apart from the BHI broth that was sterilized by autoclaving, other constituents were filter-sterilized using membrane filters.

Glucose hydrolysis: Into the basal medium, 5.0ml of 10% glucose (w/v) and 0.5ml of 0.5% phenol red were incorporated and dispensed in aliquots of 2.0ml into screw-capped bottles. 0.5ml of the test isolates were inoculated into the tubes. An uninoculated and inoculated media without substrate were used as controls. All tubes were incubated at 37°C aerobically and read daily for 14 days. A positive result was recorded when a change in colour from pale amber to yellow was observed. No change in colour indicated a negative result. The control tubes remained unchanged.

Arginine hydrolysis: This is as described for glucose above but 5.0ml of 10% arginine was used as substrate. A positive test was recorded when a change in colour from red to a deeper red solution was observed. Both controls tubes remain unchanged.

Oxidation and fermentation test: This was performed on the glucose positive isolates. The brain heart infusion agar was used in place of the broth and all other components were incorporated as described for glucose above. The tubes were set up in duplicates and inoculated with 0.05ml of the test inocula. Two sets of controls were set up. One set was incubated aerobically; the other was overlayed with sterile Vaseline. Incubation was at 37°C and tubes were read daily for 5 days. Presence of acid production in both the aerobic and anaerobic bottles is indicative of fermentative organisms while acid production only in the aerobic bottles indicated oxidative organisms.

Film and spot test: The mycoplasma agar used for this test contains twice the normal concentration of horse serum. Onto this medium the test organisms were inoculated (by flooding the plates and discarding excess). These were incubated for 10 days at 37°C in a candle jar and the plates were examined daily during incubation for the production of film and spots.

Phosphatase test: 1.0ml of 1.0% phenolphthalein diphosphate was incorporated into the basal medium containing 20% horse serum. This was dispensed in 4.0 ml amounts (in duplicate) and inoculated with drops of the test inocula. Incubation was in air at 37°C. Uninoculated tubes were used as controls. On the 3rd day, a drop of 5N NaOH was incorporated into each of the 1st set of tubes/control and observed for an immediate change of colour to intense pink. This procedure was repeated on the 7th day with the 2nd set of tubes.

Tetrazolium reduction test: 1.0ml of 1% 2, 3, 5 triphenyltetrazolium chloride was incorporated into the basal medium and dispensed in aliquots of 3.0 ml into duplicate sterile tubes. Dense agar block cultures of test organisms were incorporated into these tubes. The first sets of tubes were covered with sterile gauze stoppers and the second sets were overlayed with 0.75ml of sterile Vaseline-paraffin mixture. Incubation was at 37°C for 7 days. The tubes were observed daily for the presence of a red or pink colour in the medium especially in the area of the agar block.

Serum digestion: Into each of the basal medium dispensed in 2.0 ml amount in screw-capped bottles, a drop of the test inocula was inoculated. A strip of used black and white photographic film (10 by 5mm) sterilized by autoclaving was incorporated into each bottle. An uninoculated medium with film was set up as control. All were incubated aerobically at 37°C and examined daily for 7 days. Positive

tests were seen as the presence of dark coloured deposit at the bottom of the bottles.

Haemolysis test: The haemolytic activity of the isolates was tested for by using the method described by Alluoto *et al* [6]; however, both sheep and human blood were tested for in this study.

Serological studies: The growth inhibition technique was used [18].

Materials

Five [5] different human mycoplasma antisera (obtained from the Dept. of Pathbiology, College of Veterinary Medicine, University of Florida, USA) were used for the serological analysis of the mycoplasma isolates. These antisera with their strain and code numbers are as shown below:

Antiserum	Strain	Code No.
M. hominis	PG21	HRC 6-46-S
M. genitalium	G37	R 822-823
M. penetrans	GTU54	R 938 – 939
M. fermentans	PG18	M B713-50-069
M. fermentans	PG18	HRC 6-62-S-170

Method

Using the running drop technique, the test inocula were inoculated across mycoplasma agar plates and 6mm sterile filter paper discs soaked with 0.02ml of the different antisera were placed at the centre of each streak. Plates were incubated aerobically at 37°C under increased carbon dioxide tension for about 5 days and were examined daily for zones of clearing around the antisera. Zones of clearing seen around antisera drops were measured in millimeters and this represented positive result for the organism to that antiserum. Absence of inhibition zones indicated negative result for the organism to the antiserum.

Results

Digitonin sensitivity: All the tested strains were sensitive to digitonin and varying diameter zones of inhibition were obtained. This test was useful in detecting the cholesterol-requiring mycoplasmas that are sensitive to digitonin. *Biochemical tests*: The results of the biochemical tests are as shown in Table 1. Based on these, 43 (78.2%) strains were identified as *M. hominis*, 8 (14.5%) as *M. fermentans* and 4(7.3%) as *M. penetrans*. Only the *M fermentans* isolates showed atypical film-like reaction but without spots on the agar surface and were thus recorded as negative. None of the isolates were haemolytic on either of the blood types used and none possessed proteolytic activity (negative serum digestion).

Growth Inhibition test: The result of the growth inhibition test is shown in Table 2. Only 13 (23.6%) strains were inhibited by *M.hominis* strain PG21. Other identified Mycoplasma strains include 4 (7.3%) *M. fermentans* PG18 serotype 6-62-S-170; 4 (7.3%) *M. fermentans* PG18 serotype B 713-501-069; and 4 (7.3%) *M. penetrans* GTU 54. None was inhibited by the *M. genitalium* antisera.

Table 1: Biochemical activity of the isolates

Test	No. positive	M.hom.	M.pen.	M. ferm.
		Isolates		
Glucose breakdown	12	0	4	8
O-F	12	0	4	8
Arginine hydrolysis	55	43	4	8
Film and Spot	0	0	0	0
Phosphatase	8	0	4	4
Tetrazolium (Ae/An)	8	0	4/4	0/4
Gelatin hydrolysis	0	0	0	0
Serum digestion	0	0	0	0
Haemolysis (hum/Sh)	0	0	0	0

Key:
M.hom. = *Mycoplasma hominis*
M.ferm. = *Mycoplasma fermentans*
M. pen. = *Mycoplasma penetrans*
(hum/sh) = human/sheep
(Ae/An) = Aerobic/ Anaerobic
- = Negative

Table 2: Growth inhibition test of the mycoplasma species.

Mycoplasma species	No. isolated	No. inhibited
M.hominis	43	13
M.fermentans 1	4	4
M.fermentans 2	4	4
M.penetrans	4	4
M. genitalium	0	0

Key:
M. fermentans serotype 1 =HRC 6-62-S-170
M. fermentans serotype 2 = MB 713-501-069

Discussion

The biochemical results showed that 78.2% of the tested isolates were identified as *M. hominis* and arginine hydrolysis was the main identifying test. Other workers also reported this as the main identifying test of *M.hominis* [6,20,21]. For *M. fermentans* strains, apart from glucose breakdown and arginine hydrolysis, they showed some variations with the reduction of tetrazolium chloride and phosphatase activities. Ozcan and Miles [22] reported that the ability to utilize glucose, fructose and

acetylglucossamine differentiated *M. fermentans* strains thus supporting the fact that *M. fermentans* strains differ in their biochemical reactions to substrates. Fabricant and Freundt [23] also reported that the reaction of various mycoplasma strains to tetrazolium chloride varied from culture to culture and also with the composition of the medium.

Four strains in this study were identified as *M. penetrans*. In addition to fermenting glucose and hydrolyzing arginine, they also possess phosphatase activities and reduced tetrazolium chloride aerobically and anaerobically. Their confirmation in this study were based mainly on their been inhibited by *M. penetrans* antisera used in the growth inhibition test. *M. penetrans* is among the emergent genital mycoplasma species [13] and has been associated along with other mycoplasmas as co-factors in Acquired Immune Deficiency Syndrome [24].

Of the 55 mycoplasma strains subjected to the growth inhibition (serological) test, only 13 (23.6%) were inhibited by the *M. hominis* PG 21 antiserum and these 13 (30.2%) were out of the 43 strains biochemically characterized as *M. hominis*. This goes to show that the remaining 30 *M. hominis* strains were not serotypable by the *M. hominis* PG21 antisera available for this study. They probably belong to other serotypes. Mycoplasmas, especially *M. hominis* have been shown to possess heterogenic properties with each strain having its own specific antigen that presumably accounts for type specificity [25]. Such antigenic heterogeneneity appears to be correlated with differences between membrane antigens or membrane proteins of individual strains [21]. Thus, Lin [25] reported that about 8 serologically different strains of *M. hominis* exist. Most recently too, Petterson *et al* [26] determined the phylogeny of the *M. hominis* clusters of the hominis group and revealed that the cluster currently comprises 19 species.

The need to use more than one antiserum in the growth inhibition test was observed in the result obtained with the *M. fermentans* strains in this study. This test was found useful in separating the 8 *M. fermentans* PG 18 strains into 2 separate serotypes – HRC 6-62-S-170 and M B713-501-069 (four strains per serotype). This was made possible because 2 serotypes of *M. fermentans* antisera were available for the test. None of the tested strains was inhibited by the *M. genitalium* antisera. This species has been reported to be highly fastidious and difficult to grow culturally [27].

This report on the biochemical characteristics of the mycoplasmas showed variations among them. This may be due to factors like techniques, environmental factors, cultivation media and even the organism strains themselves. Biochemical tests alone may not be enough to fully identify mycoplasmas hence the need of other identification methods like serological and molecular techniques may be useful. Since genital mycoplasmas are serologically heterogenous, it was reasonable to anticipate that certain serotypes might be particularly associated with certain diseases [28]. Serologic tests make it possible to serotype isolates so as to determine whether a particular serotype is associated with a particular disease. These tests are thus necessary not only in identifying strains of mycoplasmas but also in confirming the identity of the strains biochemically characterized. Identifying genital mycoplasmas to their serotypes is necessary as this will help in studying the epidemiology of the genital mycoplasmas as disease-causing agents. This study has therefore helped in establishing the presence of other species of mycoplasmas present in the genital tracts of adult Nigerians that may be associated with genital tract infections. However, for effective growth inhibition technique or any other serological tests, it is recommended that multiple antisera be used in order to identify as many serotypes as are possible.

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