

EPISOME PROFILES AND MOBILIZABLE BETA-LACTAMASE PLASMID IN *HAEMOPHILUS DUCREYI*

Virapong Prachayasittikul¹, Ratana Lawung² and Leif Bulow²

¹Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University, Bangkok 10700, Thailand; ²Department of Pure and Applied Biochemistry, Chemical Center, Lund University, Lund, S22100, Sweden

Abstract. Chancroid caused by *Haemophilus ducreyi* has been described as a significantly predisposing factor of HIV heterosexual transmission in an endemic region of both diseases. The fastidious, *H. ducreyi* has been reported world wide with various antimicrobial susceptibility patterns. A high tendency of drug resistances has generally been found among isolates derived in Thailand. In this study, the plasmids of *H. ducreyi* were isolated and analysed from 63 clinically derived organisms. Twenty-nine out of 63 isolates (46%) revealed the same plasmid profiles. Plasmid DNA was further cloned into *Escherichia coli* and transformants were selected. A 3.6 kb plasmid (pCb) carrying ampicillin resistance was subsequently identified. The pCb conferred resistance to various beta-lactam antibiotics including penicillin G, carbenicillin, piperacillin, cefazolin, cefoperazone, ampicillin-sulbactam, and amoxicillin-clavulanate but not to cefoxitin. Co-resistance to streptomycin, chloramphenicol and tetracycline was not detected. Beta-lactamase gene was located on the major pCb fragment of *EcoRI* and *AatII* cutting.

INTRODUCTION

Haemophilus ducreyi is the etiological agent of the sexually transmitted disease (STD) chancroid or soft chancre. This STD is prevalent in developing countries, especially among genital ulcerative patients (Bogaerts *et al*, 1998; Le Bacq *et al*, 1993; Taylor *et al*, 1984). Ulcerative STDs have been epidemiologically linked to the heterosexual transmission of the human immunodeficiency virus (HIV) in areas where both diseases are endemic (Jessamine and Ronald, 1990; Nelson *et al*, 1997; Wasserheit, 1992). Furthermore, individuals infected with HIV are less responsive to standard antibiotic treatment for chancroid (MacDonald *et al*, 1989; Tyndal *et al*, 1993).

Common antimicrobial agents; chloramphenicol, tetracycline, sulfonamides, kanamycin, ampicillin, and co-trimoxazole have been successfully used previously for treatment of chancroid (Hammond *et al*, 1978; Sloomans *et al*, 1987). Increasing resistance of *H. ducreyi* to antimicrobial agents has been reported from many parts of the world (Coovadia *et al*, 1985; Sng *et al*, 1982). Taylor *et al* (1985) and Rutarugsa *et al* (1990) have reported resistance to tetracycline, sulfonamide, kanamycin, and trimethoprim of clinical *H. ducreyi* isolates in Thailand. Moreover, all *H. ducreyi* isolates

have produced beta-lactamase as determined by hydrolysis of a chromogenic cephalosporin. Furthermore, it has been shown that the degree of antimicrobial resistance found in Thailand is higher than that reported for *H. ducreyi* isolates in other geographic regions (Dangor *et al*, 1990; Rutarugsa *et al*, 1990; Taylor *et al*, 1985). Many kinds of resistance plasmids of the chancroidal agent have been reported from various parts of the world (Bilgeri *et al*, 1982; Brunton *et al*, 1979; Hansfield *et al*, 1981; Totten *et al*, 1982). In this report we have classified the clinical isolates of *H. ducreyi* by determining extra-chromosomal DNA profiles and partially characterizing the mobilizable ampicillin resistance plasmid in *H. ducreyi*.

MATERIALS AND METHODS

Bacterial strains

Haemophilus ducreyi isolates were collected from chancroid patients during 1982-1983 (31 isolates) and 1989-1990 (32 isolates). They were stored in 10% skimmed milk with 20% glycerol and 5% fetal bovine serum at -80°C. They were cultured for 48 hours on chocolate agar (Gonococcal base medium supplemented with 2% bovine hemoglobin and 1% isovitalax). The medium was incubated at 33°C in a candle jar and moisture.

Beta-lactamase production

The production of beta-lactamase was detected

Correspondence: Virapong Prachayasittikul, Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University, Bangkok 10700, Thailand.

using the chromogenic cephalosporin (nitrocefin, Oxoid) substrate. Penicillinase producing *Neisseria gonorrhoeae* (PPNG) and non PPNG were used as controls.

Preparation of plasmids

Plasmids were prepared by two methods depending on the bacterial species. Plasmids of *E. coli* transformants were extracted using a QIAprep Spin Miniprep kit™ (QIAGEN). *H. ducreyi* plasmids were isolated according to Meyers *et al* (1976). *H. ducreyi* cells were harvested, washed and suspended in 25% sucrose in Tris-EDTA pH 8.0 containing 5 mg/ml lysozyme. The mixture was then gently lysed with 1/3 volume of 0.25 M EDTA pH 8.0 and 10% SDS (final concentration 1%). To the viscous solution, 5 M NaCl was added to give a final concentration of 1 M and placed at 4°C overnight. The suspension was then centrifuged at 10,000g for 30 minutes. Suspended DNA was treated with RNaseA (final concentration 25 mg/l) and further purified by phenol/chloroform extraction. DNA was analyzed on 0.7% agarose gel electrophoresis.

Transformation

Escherichia coli strain TG1 was cultivated in LB medium and transformed using the conventional calcium chloride method (Sambrook *et al*, 1990).

Susceptibility test

Antimicrobial susceptibility was tested using a disc diffusion method for the *E. coli* transformants (Lorian, 1991). The obtained ampicillin resistant transformants were tested against many kinds of beta-lactam antibiotics (Difco); cefazolin (30 µg),

cefoperazone (75 µg), piperacillin (100 µg), carbenicillin (100 µg), cefoxitin (30 µg), ampicillin-sulbactam (10 µg/10 µg), amoxicillin-clavulanate (20 µg/10 µg), respectively. Moreover, they were also tested against other groups of antibiotics; chloramphenicol (30 µg), tetracycline (30 µg), and streptomycin (10 µg).

RESULTS

We followed the method of Meyers *et al* (1976) to isolate *H. ducreyi* plasmids. All 63 clinically derived *H. ducreyi* isolates carried at least three kinds of plasmids, and plasmids from individual organisms could be classified into seven plasmid patterns (Table 1, Fig 1). The 3.6 and 7.1 kb plasmids were found in all *H. ducreyi* isolates. Pattern VI was the most prevalent (46%). However, disparity of plasmid pattern carrying between isolates collected during 1982-1983 and 1989-1990 was observed (Table 2). During 1989-1990, only patterns I, III, V, VI were found with the frequency of 3.1%, 43.8%, 3.1%, and 50%, respectively. During 1982-1983, all patterns were found with the frequency of 12.9% (Pattern I), 6.5% (Pattern II), 12.9% (Pattern III), 19.4% (Pattern IV), 3.2% (Pattern V), 41.9% (Pattern VI), and 3.2% (Pattern VII). More importantly, all seven patterns remained their unique self-individual patterns after digestion with restriction enzymes. With the three endonucleases (*Hind*III, *Pst*I, and *Eco*RI), classification of the seven plasmid patterns remained the same (data not shown).

Mobilized plasmids of *H. ducreyi* plasmid pattern VI could be transferred by artificial transformation and expressed in *E. coli* transformants.

Table 1
Plasmid patterns found in clinical *H. ducreyi* isolates.

Pattern	Plasmid size (kb)*	No. of isolates(%) (n=63)
I	3.6, 4.3, 7.1, 9.6	5(7.9)
II	3.6, 4.3, 6.2, 7.1, 9.6	2(3.2)
III	3.6, 4.3, 7.1, 8.7, 9.6, >23.1	18(28.6)
IV	3.6, 4.3, 7.1, 9.6, 10.7, >23.1	6(9.5)
V	3.6, 7.1, 8.7	2(3.2)
VI	2.1, 3.2, 3.6, 4.3, 6.2, 7.1, 9.6	29(46.0)
VII	3.6, 4.3, 6.2, 7.1, 8.7, 9.6, 10.7, >23.1	1(1.6)

* Size of plasmids were compared to λ *Hind*III fragments.

Table 2
Prevalence of *Haemophilus ducreyi* carrying plasmid patterns during 1982-1983 and 1989-1990.

Plasmid pattern	No. of isolates(%)	
	1982-1983 (n=31)	1989-1990 (n=32)
I	4 (12.9)	1 (3.1)
II	2 (6.5)	0 (0)
III	4 (12.9)	14 (43.8)*
IV	6 (19.4)*	0 (0)
V	1 (3.2)	1 (3.1)
VI	13 (41.9)	16 (50.0)
VII	1 (3.2)	0 (0)

*Denote significant difference between the two time periods.

These plasmids could be successfully maintained in the *E. coli* host under ampicillin selective pressure. The ampicillin resistance transformants produced beta-lactamase enzymes which were secreted into the medium. The transformants were susceptible to cefoxitin, but were resistant to many other kinds of beta-lactam antibiotics including cefazolin, cefoperaxone, piperacillin, carbenicillin, ampicillin-sulbactam, and amoxicillin-clavulanate, respectively. However, they were susceptible to other groups of antibiotics such as chloramphenicol and streptomycin. They were found to possess the 3.6 kb plasmid (pCb). This plasmid could not be digested by *Nsi*I, *Not*I, *Sac*I, and *Sma*I restriction enzymes while provided certain site for abundance of restriction endonuclease (Fig 2). We found that ampicillin resistance gene is located on the major fragment of pCb after digestion with *Eco*RI and *Aat*II.

DISCUSSION

The fastidious *H. ducreyi* has been reported world wide with various antimicrobial susceptibility patterns (Coovadia *et al*, 1985; Sng *et al*, 1982). Plasmids of similar molecular weights have been identified from epidemiologically linked cases and plasmids of different molecular weight have been found in isolates from different geographic areas (Bilgeri *et al*, 1982; Brunton *et al*, 1979; Hansfield *et al*, 1981; Totten *et al*, 1982). Resistance plasmids of the chancroidal agent have been reported

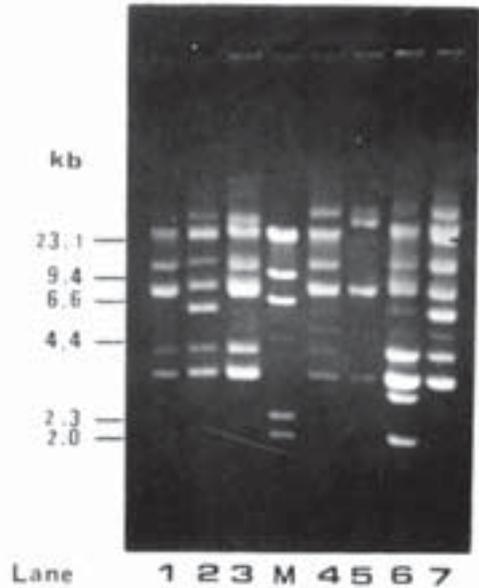


Fig 1-Plasmid profiles of clinical *H. ducreyi* isolates: Lane 1, 2, 3, 4, 5, 6, and 7 represented the plasmid pattern I, II, III, IV, V, VI, and VII, respectively, as classified from 63 *H. ducreyi* isolates. Lane M is λ *Hind*III fragments as molecular weight markers.

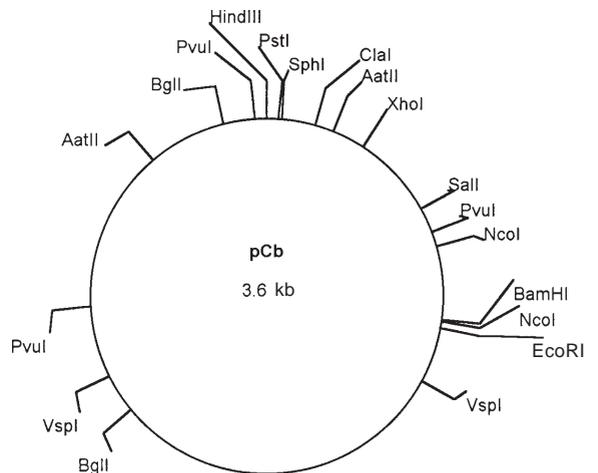


Fig 2-Restriction enzymes map of pCb ampicillin resistance plasmid.

from many parts of the world. We found that every single isolate of *H. ducreyi* carried at least three plasmids. Such plasmids may be cryptic, carry the unreveal function or carry drug resistance characteristics. Using plasmid sizes as criterion of cell classification we could subtype the *H. ducreyi* into seven plasmid profile types (Table 1). More-

over, disparity of plasmid patterns carrying between isolates collected during 1982-1983 and isolates collected during 1989-1990 suggested a mobility in plasmid compositions (Table 2). Our results are also similar to those previously described by Sarafian *et al* (1991). They found that 29 out of 30 *H. ducreyi* isolates possessed at least three plasmids, as determined by the distribution of molecular mass observed. Possible eight plasmids, ranging in size from about 1.8 to 10 Mda, were observed. The frequency of plasmids found in Thailand is much higher as compared with other regions. The isolates from Thailand appear to be unique; the number and diversity of the plasmids present in each of these isolates distinguish them from strains previously isolate in United States, Canada, or Kenya (MacNicol and Ronald, 1985). In the United States, Sarafian and Knapp (1992) has reported that 30% of *H. ducreyi* isolates possessed no plasmid, and 64% possessed one plasmid and only 1 out of 342 isolates from a patient in San Francisco, California, possessed 4 plasmids. This also indicates the potential of plasmid pattern as a molecular epidemiological tool. Although, it has been suggested that the molecular weight plasmid profile is not the epidemiological marker of strain identity (Handsfield *et al*, 1981; Totten *et al*, 1982). However, plasmid profiles are widely accepted and used as simple epidemiological marker in many kinds of bacterial infections (Olsen *et al*, 1992).

Mobilization of extrachromosomal DNA, particular R plasmid of *H. ducreyi*, to other gram negative bacteria has previously been reported (Brunton *et al*, 1986). Deneer *et al* (1982) have demonstrated an African *H. ducreyi* isolate containing three plasmids; a 10.6 kb ampicillin resistance plasmid, a 7.4 kb sulfonamide resistance plasmid, and a 35.6 kb phenotypically cryptic plasmid. In natural event of genes transforming the 35.6 kb can mediate the transfer of non-conjugative 10.6 kb ampicillin resistance gene to the recipient bacteria by transconjugation. Transferring of beta-lactamase gene to *H. parainfluenzae* and *N. gonorrhoeae* have been reported (Brunton *et al*, 1986; MacNicol *et al*, 1986). Variant ampicillin resistance plasmids have been reported from many geographic regions; the 10.6 kb, the 8.6 kb which is prevalence in African isolates, the 4.8 kb which is prevalence in US and Canada isolates, the 5.4 kb, the 3.9 kb which is reported from Thailand (Brunton *et al*, 1979; Handsfield *et al*, 1981; Maclean *et al*, 1992; MacNicol and Ronald, 1985; Sarafian *et al*, 1991; Totten *et al*, 1982). Our artificial gene

transfer of *H. ducreyi* plasmids to *E. coli* revealed the 3.6 kb ampicillin resistance plasmid which is digested by many restriction endonucleases. This plasmid may be identical to the 3.9 kb plasmid that was described by Sarafian *et al* (1991). However, the ampicillin resistance transformants of *E. coli* were not obtained when the donor DNA contained the 3.9 kb plasmid. It will be interesting to further classify and characterize the beta-lactamase enzyme which is produced by the ampicillin resistance transformant of *E. coli* carrying the 3.6 kb plasmid.

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