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Phenotypic Stability and Plasmid Detection in Actinobacillus actinomycetemcomitans

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The stability of hemolytic activity, antibiotic resistance and plasmid detection in Actinobacillus actinomycetemcomitans isolates were studied. These characteristics were stable for all experimental conditions. All tested isolates lost or changed some phenotypic characteristics such as colonial morphology and growth in liquid medium.

Key Words: Actinobacillus actinomycetemcomitans, stability, hemolysis, antimicrobial susceptibility, plasmids.

INTRODUCTION

Actinobacillus actinomycetemcomitans is a rod-shaped Gram negative coccobacilli, capnophilic, which plays an important role in localized juvenile periodontitis and in other non-oral infections such as septicemia, meningitis, endocarditis, lung and brain abscesses. Some strains of A. actinomycetemcomitans produce a potent leukotoxin as a major virulence factor that destroys human polymorphonuclear cells (1,2).

Studies on hemolysis and antimicrobial susceptibility in Escherichia coli have shown that bacterial subcultures and/or stocks can induce either loss or expression of these factors (3,4). Many bacteria are known to harbor small extrachromossomal circular DNA molecules or plasmids which may codify several phenotypic traits such as resistance to antibiotics, toxin production, hemolysin production and surface antigen. No studies of A. actinomycetemcomitans have reported the stability of these properties under environmental pressure such as continuous subcultures, changes in temperature or under action of mutagenic substances.

The goal of this study was to examine the hemolytic activity, antimicrobial susceptibility and plasmid detection in A. actinomycetemcomitans isolates, during continuous subcultures and growth at different temperatures.

MATERIAL AND METHODS

Sixteen A. actinomycetemcomitans isolates were isolated from patients with adult periodontitis (age, 20-40 years) at the Periodontic Clinic of the School of Dentistry, University of São Paulo, SP, Brazil. Samples were taken from 20 patients (12 males, 8 females) who exhibited clinical and radiographic evidence of alveolar bone loss and periodontal pocket depth ≥5 mm. None of the patients had received antibiotics for at least 3 months prior to sample collection.

Briefly, supragingival dental plaque was removed from the mesial surface of 2 teeth using sterile cotton pledgets. Subgingival bacterial samples were taken from the periodontal pockets (5) and then transferred to tubes containing 2.0 ml of prereduced anaerobically sterilized Ringer solution, pH 7.2. Samples were cultured 2 h after collection. After dispersion by a vortex mixer, 0.1 ml of undiluted to 10^{-3} diluted sample solutions were plated on a selective trypticase soy serum bacitracin vancomycin (TSBV; Difco, Detroit, MI, USA) agar (6). After 72 hours of incubation at 37°C, under anaerobic conditions (90% N_2/10% CO_2), suspected colonies were subcultured and then identified as A. actinomycetemcomitans if they were Gram-negative coccobacilli and produced translucent colonies with a starlike inner structure, 0.5 mm to 1.0 mm in diameter and catalase,

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and if they did not ferment lactose, starch, sucrose and trehalose (7). Two reference strains A. actinomycetemcomitans ATCC 29523 and FDC Y4 were also included. All isolates were stored in 20% glycerol at -70ºC.

Hemolysis production was performed on trypticase soy agar (Difco Laboratories) supplemented with 0.5% yeast extract and 5% human blood (A Rh+, B Rh-, O Rh+ or AB Rh+ groups). Twenty µl aliquots of each isolate (approximately 10^7 cells/ml) were plated and incubated at 37ºC for 48 h, under microaerophilic conditions. Inocula size was verified by colony count.

The minimal inhibitory concentration (MIC) for clindamycin, penicillin G, erythromycin, lincomycin, metronidazole, and tetracycline was determined with an agar dilution method (8), on brain heart infusion (BHI, Difco) agar supplemented with 0.5% yeast extract. Plates were inoculated with a Steers replicator (approximately 10^4 cells/spot). Inocula size was verified by colony count.

In order to study the bacterial stability, initial cultures, stored at -70ºC, were grown in BHI broth and were then subcultured 50 times in BHI broth supplemented with 0.5% yeast extract at one or two day intervals, as visible growth occurred (10^7 cells/ml). Hemolytic activity and antimicrobial susceptibility were tested every 10th subculture. Bacteria were cultured in BHI broth supplemented with 0.5% yeast extract, incubated under microaerophilic conditions (candle method), at 37ºC, for 48 h, and 0.2 ml was transferred to fresh BHI broth and incubated at 30ºC, 37ºC and 40ºC, under similar conditions, for 48 h. After growth at these temperatures, hemolysis and antimicrobial susceptibility were tested. No growth was detected when incubated at 40ºC. Temperatures were chosen by testing an extreme temperature to bacterial growth.

Cultured isolates under similar conditions were supplemented with increasing concentrations of ethidium bromide (0.5 to 16 µg/ml EtBr). Aliquots of growing bacteria from tubes with higher subinhibitory concentration (5 µg/ml) were used to test hemolytic activity and antimicrobial susceptibility. All experiments were performed in triplicate.

Cultures of A. actinomycetemcomitans were screened for the presence of plasmid DNA by the method of Birnboim and Doly (9), modified by increasing the BHI broth volume to 50 ml. Bacteria were grown and carefully harvested from BHI broth by centrifugation, and washed twice in 0.9% saline solution. Fifty µl of the DNA sample were submitted to horizontal electrophoresis and DNA fragments were separated on a gel containing 0.5 mg/ml EtBr. The gels were run at 70 V for 2.5 h and were photographed using an ultraviolet transilluminator with an orange filter and polaroid Kodak Panatomic X film.

RESULTS AND DISCUSSION

Hemolysis has been reported in a broad spectrum of Gram-negative and Gram-positive species, and both epidemiological and experimental data have indicated its association with bacterial pathogenicity (4).

The MIC values for the A. actinomycetemcomitans isolates can be observed in Table 1. Antibiotics used are the most frequently chosen in dental treatments. Only slight variations were observed for erythromycin, lincomycin and tetracycline (two-fold resistance increase). Resistance was increased twice after the 10th subculture, but after the 40th subculture the MIC decreased to the initial values for metronidazole. For penicillin G, the MIC decreased after the 10th subculture (two-fold) remaining stable up to the 50th subculture. These variations in the MIC values could be due to cellular alterations as a consequence of stress produced by continuous subcultures. No alterations of these phenotypic characteristics were detected due to variations in growth temperature and under EtBr effects.

Table 1. Antimicrobial activity of 16 A. actinomycetemcomitans isolates at different points during subculture.

Antibiotics	Range of MIC (µg/ml) at different subculture points*					
	1st	10th	20th	30th	40th	50th
Clindamycin	4-32	4-32	4-32	4-32	4-32	4-32
Erythromycin	1-16	1-16	2-16	2-16	2-16	2-16
Lincomycin	8-128	8-128	8-128	8-128	8-128	16-128
Metronidazole	16-128	32-128	32-128	32-128	16-128	16-128
Penicillin G	8-128	4-128	4-128	4-128	4-128	4-128
Tetracycline	0.5-1	0.5-1	0.5-1	0.5-1	0.5-1	1

*Similar results were observed in growth at 30ºC, 37ºC and during subinhibitory concentration of EtBr.

All isolates were alpha-hemolytic during the subculture period. Slight quantitative alterations in the hemolytic activity were observed in relation to the diameter of the lytic halo (from 7-8 mm to 8-10 mm). On the other hand, the A. actinomycetemcomitans isolates lost some phenotypic features such as colonial morphology (adherence and starlike inner structure) and growth mode in liquid medium during subculture (growth with a granular sediment strongly adherent to the test tube bottom). This could indicate a possible instability of other phenotypic characteristics that were not studied. In the present experiment, both hemolytic activity (alpha-hemolysis) and antimicrobial susceptibility of the tested isolates were stable during the experimental period.

Two methods were tested to detect plasmid with the Birnboim and Doly (9) method being the most useful. The Kado and Liu method (10) failed to demonstrate the presence of plasmids in A. actinomycetemcomitans. Plasmid enriched lysates from isolates 55, 41 and 58 apparently contained the same plasmid of approximately 90 Kb (Figure 1). However, it is important to show that the second band observed in isolate 41 seems to be representative of another plasmid with a molecular weight of approximately 70 Kb. The isolate 30 (lane 8) showed its total DNA degraded (Figure 1).

It is unclear whether the 7 isolates in which plasmids could not be detected did not harbor them or whether the plasmids were lost during the passages. The later assumption is possible because plasmids could not be detected in some of the isolates after further culture, even when they were seen to be harbored originally. The prevalence of extrachromossomal DNA in isolates of A. actinomycetemcomitans might help in equipping them with a variety of determinants, such as virulence and drug resistance.

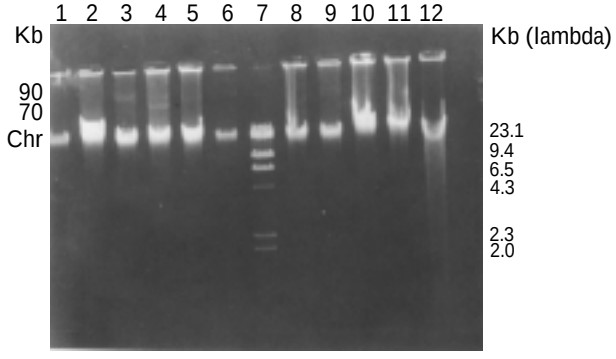

Figure 1. Agarose gel electrophoresis of plasmid DNA. Lane 1: A. actinomycetemcomitans ATCC 29523; lane 2: isolate 1; lane 3: isolate 55; lane 4: isolate 41; lane 5: isolate 66; lane 6: A. actinomycetemcomitans FDC Y4; lane 7: HindIII; lane 8: isolate 30; lane 9: isolate 58; lane 10: isolate12; lane 11: isolate15; lane 12: isolate17.

Our results did not permit an association between the presence of plasmid with the hemolytic activity or resistance to the studied drugs, because the other isolates, in which plasmids were not detected, showed both the hemolytic activity and resistance to antibiotics, before and after treatment with EtBr. Le Blanc et al. (11) suggested that the presence of plasmids among strains of oral pathogens such as A. actinomycetemcomitans is rare. Only 4 of 16 examined isolates contained detectable DNA plasmid. The absence of plasmid in most of the studied isolates is in sharp contrast to the results of Olsvik and Preus (12) who reported the presence of 3 small plasmids and the presence of large plasmids (>30 Kb) in ten isolates. Whereas most of the isolates in this study were subcultured 50 times, the plasmid-containing isolates appeared to have maintained their plasmid quite stable. In this study, we can conclude that detected plasmids must be considered as cryptic because no known phenotypic function has been ascribed to them.

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RESUMO

Avila-Campos MJ, Padilla G. Estabilidade fenotípica e detecção de plasmídios em Actinobacillus actinomycetemcomitans. Braz Dent J 2001;12(2):105-108.

Neste estudo foram avaliadas a estabilidade da atividade hemolítica, a resistência a antibióticos e a detecção de plasmídios em cepas orais de A. actinomycetemcomitans. As características foram estáveis durante os diferentes repiques sucessivos realizados nas condições do nosso experimento. Todos os isolados de A. actinomycetemcomitans testados, perderam ou mudaram algumas características fenotípicas tal como morfologia colonial e o crescimento em meio líquido.

Unitermos: Actinobacillus actinomycetemcomitans, estabilidade, hemólise, susceptibilidade a antimicrobianos, plasmídios.

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