The identification of the homology between iga (IgA1 protease) gene of *Ureaplasma urealyticum* and putative iga gene of *Mycoplasma genitalium*

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Abstrak

Ureaplasma urealyticum dan Mycoplasma genitalium adalah patogen pada membran mukosa. Keduanya berkerabat dekat, dan dimasukkan dalam familia Mycoplasmataceae. Genom U. urealyticum dan M. genitalium terdiri atas satu molekul DNA pilin ganda yang sirkuler. Dengan adanya kekerabatan yang dekat, diharapkan gen iga U. urealyticum dan gen iga putatif M. genitalium mempunyai homologi yang tinggi, sehingga penemuan ini dapat dipakai sebagai alat untuk diagnosis dan untuk mempelajari patogenesis U. urealyticum. Penelitian ini diawali dengan melakukan kultivasi U. urealyticum standar 27618 ATCC (American Type Culture Collection) di dalam medium Ureaplasma. Pertumbuhan U. urealyticum diverifikasi dengan cara mengamplifikasikan gen urease pada genom U. urealyticum dengan primer gen urease yang kompatibel, dengan teknik PCR. Hasil amplifikasi diobservasi dengan elektroforesis gel mini agarosa. Desain primer untuk amplifikasi gen iga U. urealyticum dengan teknik PCR dilakukan berdasarkan urutan nukleotida gen iga putatif M. genitalium. Hasil amplifikasi diobservasi dengan metoda elektroforesis gel mini agarosa, selanjutnya gen iga pada gel tersebut dipurifikasi dengan "glassmax", dan hasilnya dilabel dengan digoksigenin. Gen iga U. urealyticum berlabel digoksigenin tersebut kemudian dihibridisasikan dengan genom U. urealyticum, untuk mengetahui apakah gen iga yang teramplifikasi berasal dari genom U. urealyticum. Sekuensing gen iga U. urealyticum hasil amplifikasi dilakukan untuk mengetahui besarnya homologi antara gen iga U. urealyticum dengan gen iga putatif M. genitalium. Sekuensing dilakukan dengan mesin "AB1377 DNA sequencer" di Lembaga Eijkman. Dari hasil penelitian dapat disimpulkan bahwa: Terdapat homologi 100% antara gen iga U. uralyticum dan gen iga putatif M. genitalium sepanjang 0,40 kb.

Abstract

Ureaplasma urealyticum and Mycoplasma genitalium are mucosal pathogens which are closely related. They both belong to the family Mycoplasmataceae. The genomes of U. urealyticum and M. genitalium consist of one molecule of circular double-helix DNA. This study was performed to evaluate whether the iga gene of U. urealyticum has high homology with the putative iga gene of M. genitalium, so that this finding might be used as a tool for diagnosing and studying the pathogenesis of U. urealyticum infection. First, cultivation of U. urealyticum in the Ureaplasma media and isolation of the genome were done. Next, verification of the growth of U. urealyticum by amplification of the urease gene of U. urealyticum genome using PCR technique (Kui Teng, 1994). The PCR product was observed by agarose mini gel electrophoresis. DNA primers for amplifying U. urealyticum iga gene were designed based on the nucleotides sequences of putative iga gene M. genitalium. The primers were used for amplifying U. urealyticum iga gene with PCR technique, using the genome of U. urealyticum as the template. The PCR product was observed by agarose mini gel electrophoresis. The iga gene of U. urealyticum was purified by the glassmax method and then labeled with digoxigenin. The digoxigenin labeled iga gene was hybridized to the genome of U. urealyticum, to find whether the iga gene amplified was derived from the genome of U. urealyticum. Sequencing the PCR product of the U. urealyticum iga gene was done to evaluate the degree of homology between U. urealyticum iga gene and M. genitalium putative iga gene using "AB1377 DNA sequencer" in the Eijkman Institute. Conclusion of this study : There is 100% homology between a 0,40 kb fragment of U. urealyticum iga gene and a 0,40 kb fragment of M. genitalium putative iga gene.

Keywords:*cultivation, electrophoresis, hybridization, sequencing.*

U.urealyticum and *M. genitalium* are mucosal pathogens which are closely related. They are both classified in the class of *Mollicutes* and belonged to the order of *Mycoplasmatales*, family of *Mycoplasmataceae*. *U. urealyticum* belonged to the genus *Ureaplasma*, while *M. genitalium* belonged to the genus *Mycoplasma*. Their genomes are a single circular double stranded DNA molecule (5 X 108 daltons), in an unbounded nucleus, and contains low G+C.[6,9] *U. urealyticum* is a gram negative bacteria, small (200-250 nm diameter), nonmotile, and has no

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rigid cell wall. While *M. genitalium* is a gram positive bacteria, small (200-250 nm diameter), nonmotile, and has no rigid cell wall.[9]

U. urealyticum and *M. genitalium* have been recovered in humans; they efficiently colonize and infect the mucous membrane of the lower genital tract of women and upper genital tract of men. Those infections have been associated with nongonococcal urethritis, salphingitis, and infertility.[6,9] McCormack,[10,11] and Poulsen[15] demonstrated that rates of genital tract colonization of *U. urealyticum* in men and women varied from 3% to 56% and 8,5% to 77,5% respectively depending on age, race, socioeconomic status, and sexual experience. Thus, *Mycoplasmas* and *Ureaplasma* are indeed sexually transmitted. Tjokronegoro[18] suggested that colonization of *U. urealyticum* in the male semen of infertile couples does not interfere the sperm motility. However, its effect on the ability of the spermatozoa to fertilize the ovum has not been ruled out, since *U. urealyticum* can adhere to the acrosome of the spermatozoa. The presence of *U. urealyticum* in the lower and upper female genital tract and in the central nervous system of the newborn has also been associated statistically to the prematurity, low birth weight infants, infertility, morbidity, and mortality of the newborn.[1,3,6,12,13,16,17,19]

U. urealyticum produces IgA1 protease.[5] IgA1 protease is extracellular enzyme which can hydrolizes IgA1 (immunoglobulin A1). IgA1 protease specifically cleaves the IgA1 isotype at a single Pro-Thr or Pro-Ser peptide bond in the hinge region of immunoglobulin, releasing intact Fc and Fab fragments.[5] IgA1 proteases enzyme are also released by pathogens such as *Neisseria meningitidis, Haemophilus influenzae*, and *Streptococcus pneumoniae*, those cause the bacterial meningitis, pneumonia, gonorrhea, urinary tract infections, periodontal disease, and dental plaque. Since IgA is the predominant immunoglobulin on the mucous membrane, the IgA proteases may be important virulence factors for pathogen organisms to entry the mucous membrane. Thus, these IgA-specific proteases have capability to destroy the pathogen-specific antibody activities.[5,8]

N. meningitidis, H. influenzae, and *S. pneumoniae* contain iga gene that codes the IgA1 protease.[8] On the other hand, *M. genitalium* that can colonize and infect the mucous membrane of human organs has nuclotides sequences (putative iga gene) which is homologous to the iga gene of *H. influenzae*.[4]

The purpose of this study is to identified the homology between iga gene of *U. urealyticum* and putative iga gene of *M. genitalium* and to evaluate whether this finding can be used as a tool for diagnosing and studying the pathogenesis of *U. urealyticum infection.*

MATERIAL AND METHODS

This study used *U. urealyticum* standard 27618 obtained from American Type Culture Collection (ATCC).

Cultivation

First, cultivation of *U. urealyticum* in the *Ureaplasma* broth was done by Shepard method with modification,[9] as follows: *U. urealyticum* standard 27618 were grown in the 200 mL of *U.* broth which had been adjusted to pH 6.0. That *U.* broth contained 1% *Mycoplasma* broth base (Difco), 0,1% Yeast extract (Difco), 2 mL of 4% bromo thymol blue or phenol red, 10% horse serum, 0,5 mL of 10% urea, 160 mL ddH_2O, 1.000 IU/mL peniciline-G, and 25 µg/mL amphotericine-B. The culture was incubated aerobically for 1-7 days at 37°C. In this liquid medium, the growth of *U. urealyticum* was shown by the change of colour of the indicator to alkaline (from yellow to be red by using phenol-red indicator or green by using bromo thymol blue indicator). The *U.* agar medium was made by adding 1.5 g agar Noble (Difco) to the *U.* broth medium. The agar culture of *U. urealyticum* was incubated in a CO_2 incubator or a candle jar. The plated culture (medium) were inspected daily under 40 X magnification using light microscope. Since of the small colonies of *U. urealyticum* may be difficult to distinguish from various artifacts, those suspected *U. urealyticum* colonies must be confirmed by exploiting its ability to hydrolyze urea. Since the *U.* agar medium contains urea and phenol red, the presence of *Ureaplasma* colonies will be shown as colonies surrounded by red halo.

The suspected *U. uralyticum* colonies were also confirmed using the manganous chloride-urea test. In this test, an agar plate containing colonial growth was flooded with an aqueous solution of 1% urea and 0,8% manganous chloride. On this medium, *U. urealyticum* colonies will be appear within 1 to 3 days, 15 to 50 um in diameter, and golden brown in colour due to the accumulation of manganese oxide in the colonies.[6,9]

Isolation of the genome

Isolation of *U. urealyticum* genome was based on the Kui Teng method.[7] With modifications as follows: 1.5 mL *U. urealyticum* culture were centrifused at 11,800 X g for 60 minutes. The 1 mL of supernatant was discarded, and the remainder was suspended in 400 mL of TE (Tris-EDTA). To the solution 30 µL of 10% SDS (Sodium dodecyl sulfate) and 2.5 µl of proteinase K (2mg/mL) was added. After incubation at 50°C for 1 hour, the solution was mixed to 500 µL of saturated phenol (pH 8.0), and centrifuged at 5,000 X g for 5 minutes. The aqueous phase was harvested then mixed with 500 µL of chloroform-isoamyl alcohol (24:1) and centrifuged again at 5,000 X g for 5 minutes. The aqueous phase was transfered to the other microtube. The DNA was precipitated by absolute ethanol at -20°C for more than 2 hours /overnight, centrifuged at 5,000 X g for 5 min. The DNA was rinsed by 70% ethanol, air dried, and then dissolved in 60 µL of TE solution (pH 7.5).

The growth of *U. urealyticum* was verified by amplifying the urease gene of *U. urealyticum*. The genome of *U. urealyticum* was partially digested by restriction enzyme EcoR1 for 2 hours at 37°C, and used as the template for amplifying urease gene of *U. urealyticum* by PCR technique. The sequences of the primers are as follows: 14b, 5'-CCAGGAAAAGTAGTACCAG-GAGC-3'., and C72b, 5-CTCCTAATCTAAC-CCTATCACC-3.[21] *U. urealyticum* DNA was amplified in a DNA thermal cycler (Perkin-Elmer Cetus Corporation), during 30 thermocycles, each consisting of a 30-s denaturation step at 94°C, a 30-s annealling step at 55°C, and a 90-s elongation step at 68°C. The PCR product was visualized and photographed under UV light after electrophoresis for 50 min at 90 Volts through a 0,8% agarose mini-gel containing ethidium bromide.[2] The length of DNA fragment was determined by comparing to DNA marker.

Primer design of iga gene of U. urealyticum

DNA primers for the amplifying iga gene of *U. urealyticum* were designed based on nucleotides sequences of the putative iga gene (MG219) of *M. genitalium G37*[4] by computer. The sequences of the primers are as follows: the forward primer, 5-AAAAATACCCATAATGAATAGTGATAG-3', and the reverse primer, 5'-TTTTGTTTGAAT-TTTGGGTTGGTTTAG-3'. The iga gene of the genome of *U. urealyticum* (as the template) was amplified in a DNA thermal cycler (Perkin-Elmer Cetus Corp) during 30 thermocycles, each consisting of a 30-s denaturation step at 94°C, a 30-s annealling step at 68°C, and a 90-s elongation step at 72°C. The result of amplification was visualized and photographed under UV light after electrophoresis for 50 minutes at 90 Volts through a 0,8% agarose mini-gel containing ethidium bromide. The *U. urealyticum* iga gene was purified by the glassmax method (Gibco BRL) and then observed by electrophoresis

Hybridization

The iga gene of amplification result was hybridized with *U. urealyticum* genome. The hybridization has 2 aims: first, to know whether the iga gene amplified was from the genome of *U. urealyticum* (for verification); second, to estimate the length of the *U. urealyticum* iga gene. The hybridization was conducted with the Southern blot method.
The steps in Southern blot hybridization:

Preparation

After agarose gel electrophoresis of *U. urealyticum* genome, any unused area of agarose gel was trimed away. Depurinization of DNA was performed by soaking the gel in the solution A (0,25 M HCl) for 15 min; denaturation of DNA was done by soaking the gel in the solution B (0.5 NaOH; 1.5 NaCl), and neutralization of DNA in the solution C (1,5 M NaCl; 0,5 M TrisHCl; 0,001 M Na_2EDTA) pH 7.2. for 2 X 30 min. at room temperature.

Southern blot transfer

Two pieces of 3 MM Whatman papers (which are longer and wider than the agarose gel), were immensed into dish filled with 10 X SSC (Saline Solution Citrate). The agarose gel was placed on the damp Whatman papers, and made sure no air bubbles between them. Hybond nylon as wide and as long as the gel was placed on the gel. Three pieces of damp Whatman papers were placed on the hybond nylon. A stack of paper towels was placed on the Whatman paper, and a 0.5 kg burden was placed on the top. Transfer of the DNA bands from agarose gel to the hybond nylon was allowed overnight. Southern blot transfer results in a hybond nylon that carries a replica of the DNA bands from the agarose gel.[2] The replica of the DNA bands was then hybridized with the *U. urealyticum* iga gene as follows:
First, hybond nylon was soaked in the 20 mL prehybriditation solution (5 mL of 5 X SSC, 40 µL of 0,02 % SDS, 2 mL of 1% blocking reagent, 200 µL of

0,1% N-Lauryl sarcosine, and 12.760 mL dH_2O), at 65°C, for 2 hours. *U. urealyticum* iga gene of amplification result after labeling with digoxigenin (Boehringer Mannheim Biochemica) was hybridized to the U. urealyticum genome in the hybond nylon, as follows: The denatured digoxigenin labeled DNA was added to the hybridization solution (the same as prehybridization solution) of hybond nylon and shaked slowly at 65°C, overnight. The hybridization was then detected as follows: The solution was discarded and hybond nylon washed by solution 1 (198 mL of 2 X SSC; 2 mL of 10% SDS) at room temperature for 2 X 5 min. The washing was continued with solution 2 (0.1% SSC; 0,1% SDS) at 65 °C for 2 X 15 min., and maleic acid buffer or buffer 1 pH 7.5 (0,1 M maleic acid, 0,15 M NaCl) at room temperature, for 5 min. The nylon was then incubated in the buffer 2 (blocking reagent:buffer1=1:10) at room temperature for 30 min., and continued in the anti-digoxigenin solution (2 µL anti-dig/18 µL buffer 2) at room temperature for 30 min. The nylon was then washed in buffer 1 for 2 X 15 min, and equilibrated by buffer 3 for 5 min. The DNA bands were droped by 100 µL CSPD and incubated at 37 0C for 15 min. The nylon was exposed with X-ray film in the dark room for 30 min., and developed it in the developing solution.for visualization of DNA bands.

Sequencing

Sequencing the *U. urealyticum* iga gene of amplification result was done by "AB1377 DNA sequencer" in Eijkman Institute.

RESULTS

The incubation periode of *U. uralyticum* was 1 to 7 days (Figures 1 to 4). Figure 1 and 2 show the growth of *U. urealyticum* in *U.* broth using phenol red (red coloured) and bromo thymol blue indicator.(green coloured) respectively. Figure 3 and 4 show the growth of *U. urealyticum* in the *U.* agar using phenol red indicator (red coloured) and manganous chloride-urea-test (golden brown coloured) respectively. Observation by agarose electrophoresis for amplification result of *U. urealyticum* urease gene showed DNA band of 0,46 kb (Fig 5). Observation by agarose electrophoresis for amplification result of *U. urealyticum* iga gene showed a DNA band of 0,40 kb (Fig. 6). The Southern hybridization between *U. urealyticum* genome and iga gene of amplification result showed several DNA bands, one of the bands is 4,8 kb in length (Fig. 7). Sequencing of *U. urealyticum* iga gene showed those nucleotides sequences of *U.*

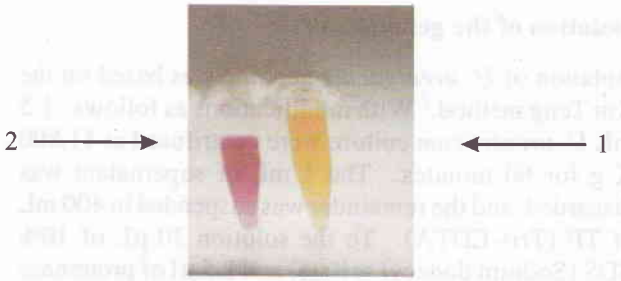

Figure 1. Liquid culture of U. urealyticum with phenol-red indivator
1. Controle: Medium without U. urealyticum (yellow)
2. Culture of U. urealyticum (red)

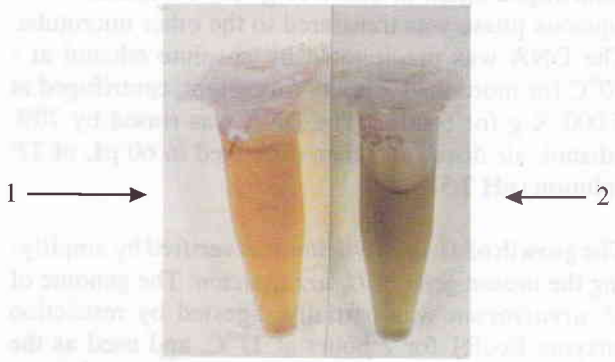

Figure 2. Liquid culture of U. urealyticum with bromothymol-blue indicator
1. Controle: Medium without U. urealyticum (yellow)
2. Culture of U. urealyticum (green)

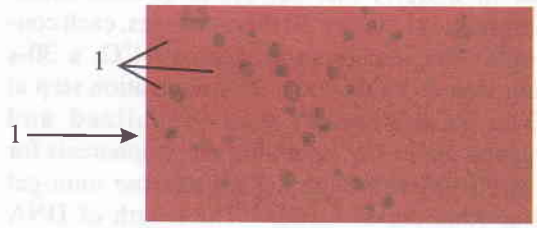

Figure 3. Solid culture of U. urealyticum (medium contains phenol-red indivator and urea)
1. Colonies of U. urealyticum (red)

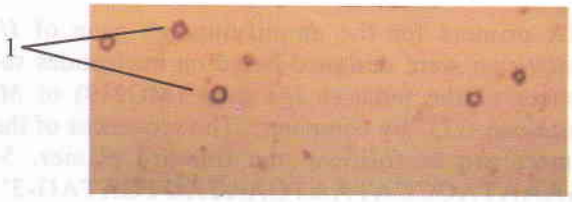

Figure 4. Manganous chloride urea-test of U. urealyticum growth of solid culture
1. MnO precipitate around the colony of U. urealyticum (golden brown)

urealyticum iga gene and *M. genitalium* putative iga gene are really the same (Fig. 8a and 8b).

DISCUSSION

Ureaplasmas are distinct from other mycoplasmas in their ability to hydrolize urea to carbon dioxide and ammonia by action of the urease enzyme. This ability is a key function of their metabolism, as inhibition of the urease or a lack of urea in the environment will arrest the growth.[21] Growth in solid or liquid medium is conveniently monitored by the change of colour of the indicator to alkaline as the result of formation of ammonia from the urea (Figures 1, 2, 3). Suspected *U. urealyticum* colonies of solid culture can also be confirmed by the manganous chloride urea-test. Hydrolysis of urea by urease liberated hydroxyl groups from water, and these hydroxyl moieties oxidized manganous chloride to insoluble manganese oxide, causing the deposition of golden brown precipitate around the colonies themselves within a few minutes (Figure 4).

The verification result of *U. urealyticum* growth by amplification of urease gene of *U. urealyticum* genome using PCR technique is positive. By agarose electrophoresis the length of fragment of *U. urealyticum* urease gene is 0,46 kb (Figure 5). The amplification of U. urealyticum urease gene used the primers which did not conserve with urease gene of other microbia. Since the primers used were specific ones and the length of the *U. urealyticum* urease gene fragment was produced by this amplification was the same as the length of urease gene fragment that was produced by Kui Teng,[7] thus that growth is really the growth of *U. urealyticum.*

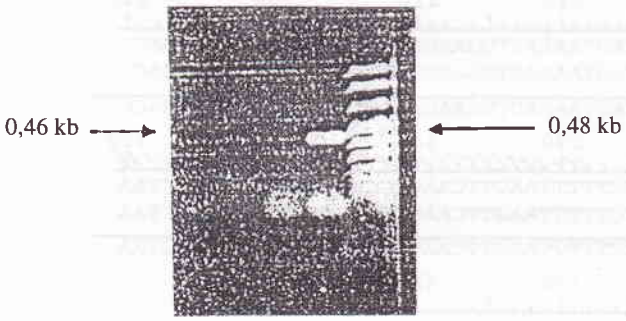

Figure 5. Electrophoresis of amplification result of U. urealyticum urease gene.
Left: urease gene (0,46 kb); right: marker VIII

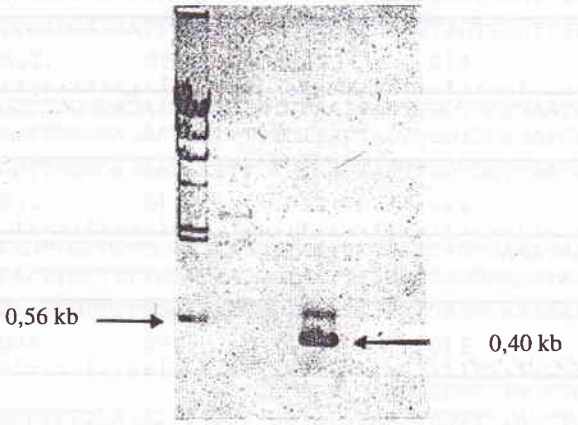

Figure 6. Electrophoresis of amplification result of U. urealyticum iga gene.
Left: Marker II; right: U. urealyticum iga gene (0,40 kb).

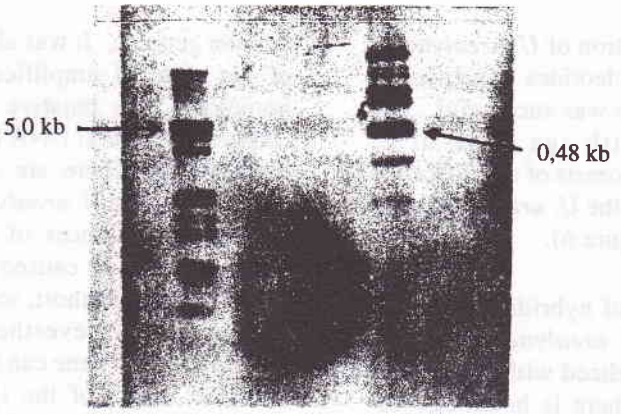

Figure 7. Hybridization between U. urealyticum genome and iga gene.
Left: marker III; right: U urealyticum iga gene (4,8 kb).

ATGCGCACCAGTTACTTGAAAAAAATACCCATAATGAATAGTGATAGTGATCTAAAACTC
CAAAAGGTGTGGATCGAGCGGCATGTTGATCAAGATGAACTTAGTTTAACAACTACTGCA
GTTGAACTTAAAAAGAGTGATGAACAAAAACCTGTTGCCATTAAAAGTAGTGACTTTATT
GGTCATGAAGAGTTAATCTCTGTTCCAGTTTTACTAATCCCAACCCCTGTTGTTAAAGAG
ATTGATCAACCAGCAGTTATTCCTCCAGTTAAAGCAAAACCAAAAGCAACTAAAAAGAAA
ACTCCTGTTAAATCAAAACCAACTAGTAAATCAACTAAACAAACAAAACCTAAACAATCC
AAGCCCAAATCAAAACAAGTTCAACAAACCAAAGCTAAACCAACCCAAATTCAAACAAAA
AAAAGCAATAAAAAAACCAGATCT

Figure 8a. Nucleotides sequences of M. genitalium putative iga gene

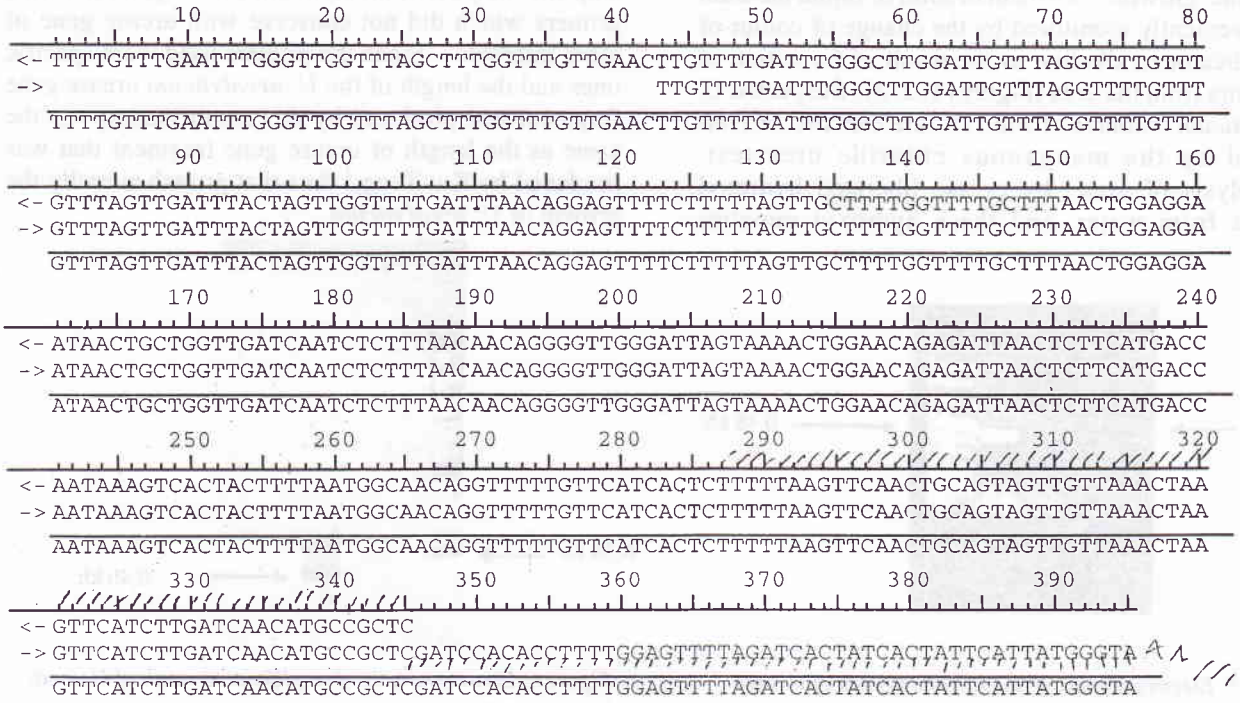

Figure 8b. Nucleotides sequences of U. urealyticum iga gene

Design of primers for amplification of *U. urealyticum* iga gene fragment based on nucleotides sequences of *M. genitalium* putative iga gene was successful. The primers are compatible with iga gene of *U. urealyticum*. Agarose electrophoresis of amplification result showed that the length of the *U. urealyticum* iga gene fragment was 0,40 kb (Figure 6).

There are several DNA bands of hybridization result (figure 7). It means: (1) The *U. urealyticum* iga gene fragment of PCR process hybridized with iga gene of *U. urealyticum* genome. (2) There is homology between nucleotides sequences of *U. urealyticum* genome and *M. genitalium* putative iga gene. (3) Amplification occured at the iga gene of *U. urealyticum* genome. It was also proved by the sequencing of iga gene of amplification result, there is 100% homology with putative iga gene of *M. genitalium* (Figure 8). Several DNA bands of hybridization result indicates that there are several sites of nucleotides sequences in the *U. urealyticum* genome homolog with nuclotides sequences of *M. genitalium* putative iga gene. It might be caused by a nucleotides chain used as probe was too short, so there are sameness with the other genes. Nevertheless the length of the *U. urealyticum* iga gene can be estimated by comparing it with the length of the iga genes of other microorganisms which have been isolated as: the length of *N. meningitidis* iga gene is 6.5 kb,8 the length of *S. pneumoniae* iga gene is 6.2 kb,[14,20] and the length of

H. influenzae HK368 is 5.1 kb.[14] Because the genome of *U. urealyticum* is smallest between eubacteria's, probably the length of *U. urealyticum* iga gene is 4,8 kb, (Figure 7), smaller than *H. influenzae* HK368 iga gene.

CONCLUSION

There is 100% homology between a 0,40 kb fragment of *U. urealyticum* iga gene and a 0,40 kb fragment of *M. genitalium* putative iga gene.

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