Review paper
Pregledni znanstveni članek

MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF AVIAN MYCOPLASMAS

P. DOVČ[a] and D. BENČINA[b]

[a] Univ. of Ljubljana, Biotechnical Fac., Zootechnical Dept., Groblje 3, SI-1230 Domžale, Slovenia, Assos.Prof., Ph.D., B.Sc.Agr.
[b] Same address, Ph.D., M.Sc., B.Sc.Vet.Med.

Received October 30, 1998, accepted November 12, 1998.
Delo je prispelo 1998-10-30, sprejeto 1998-11-12.

A B S T R A C T

Serological techniques are recommended procedures for the identification of *Mycoplasma* species. They enable separation of mycoplasmas at the species level and are widely used for practical laboratory identification of the isolated microorganisms. However, complex growth media are required for the isolation of *Mycoplasma* species and some strains of pathogenic *Mycoplasma* species grow very fastidious and can not be cultured. Introduction of DNA based diagnostic methods in mycoplasma research enabled survey of the whole genome in a single assay and reveal also information about non-coding regions of the genome. Restriction enzyme analysis is a powerful method for differentiation of close related strains but requires high quality of DNA which can only be obtained from cultured mycoplasmas. The increasing number of cloned mycoplasma genomic fragments are successfully used for identification and differentiation of mycoplasma strains and species in hybridization studies. The polymerase chain reaction enables identification and characterization of mycoplasmas without culture directly from clinical material. Large genomic sequencing projects produced complete nucleotide sequence from two *Mycoplasma* species and several more are in progress. This data will open the possibility to understand better the biology of mycoplasmas.

Key words: microbiology / mycoplasma / diagnostics / molecular genetic / DNA / polymerase chain reaction

MOLEKULARNA IDENTIFIKACIJA IN KARAKTERIZACIJA PTIČJIH MIKOPLAZEM

I Z V L E Č E K

Tradicionalne metode za identifikacijo in diferenciacijo mikoplazem temeljijo na seroloških tehnikah. Omogočajo ločevanje mikoplazem na ravni vrst in jih uporabljamo za praktično laboratorijsko identifikacijo izoliranih mikroorganizmov. Zahteva po kompleksnih medijih za izolacijo mikoplazem, njihova počasna rast in dejstvo, da nekaterih sevov patogenih vrst mikoplazem ni moč gojiti v kulturi, so glavni razlogi za uvedbo molekularnih metod v diagnostiko mikoplazemskih infekcij. Z uporabo molekularnih metod lahko zajamemo celoten genom v enem samem testu, vključno z nekodogenimi regijami. Analiza genomske DNK z restrikcijskimi encimi je zelo informativna metoda, ki omogoča razlikovanje med sorodnimi sevi, zahteva pa visoko kvalitetno DNK, ki jo lahko izoliramo samo iz mikoplazem, ki jih gojimo v kulturi. Vedno večje število kloniranih genomskih odsekov mikoplazem lahko s pridom uporabljamo za identifikacijo in diferenciacijo sevov mikoplazem v hibridizacijskih študijah. Polimerazna verižna reakcija omogoča identifikacijo mikoplazem direktno v kliničnem materialu brez predhodne kultivacije. Veliki projekti sekvenciranja genoma mikoplazem odpirajo vpogled v ustroj celotnega genoma in obetajo boljše razumevanje biologije mikoplazem.

Ključne besede: mikrobiologija / mikoplazme / diagnostika / molekularna genetika / DNK / polimerazna verižna reakcija

INTRODUCTION

Avian mycoplasma infections are economically important in poultry production world-wide. Infected flocks have reduced productivity and higher mortality of birds. The classical techniques for diagnosis of mycoplasma infections include detection of specific antibodies in sera of infected animals and isolation of mycoplasmas using appropriate growth media. The majority of hosts is infected with a number of *Mycoplasma* species, but only a few of them are pathogenic. Primary cultures frequently contain different *Mycoplasma* species and serological identification is required (Bencina and Bradbury, 1992). The cross reactivity of antibodies and sometimes a low sensitivity are the main disadvantages of serological techniques, which detect specific antibodies. Fastidious growth of pathogenic mycoplasmas under *in vitro* conditions and eventually overgrowth by faster growing *Mycoplasma* species or other organisms make isolation from clinical specimens difficult. Application of monoclonal antibodies (Mab) for the identification of isolates is critical due to the common appearance of variable expression of the immunodominant membrane proteins of mycoplasmas. Some of these problems can be eliminated by the application of highly sensitive and specific molecular techniques based on detection of nucleic acids.

The recombinant DNA technology offered a large number of analytical tools which were successfully used for the identification and molecular characterization of avian mycoplasmas. One of the major advantages of molecular techniques is the possibility of identification of genome structure which is widely independent on physiological status of the organism. Further advantage of many molecular techniques is the analysis of a number of loci spread over the entire genome at the time which enables the complex comparison among genomes of mycoplasmas. Some of these differences which are sometimes even not identifiable at the phenotypic level can be used as genetic markers for identification of mycoplasma strains. This is particularly useful in epizootiological studies where the precise differentiation among different isolates enable tracing of the infection progress. Introduction of the polymerase chain reaction (PCR) as the diagnostic tool for mycoplasma infections can help to overcome the problems related to fastidious growth of mycoplasmas under culture conditions (Razin, 1994). Further important advantage of DNA based techniques for mycoplasma identification is their capability of positive identification of new *Mycoplasma* species in contrast to the serological methods which are based on the antigenic differenciation among the candidate species belonging to the recognized *Mycoplasma* species. In this article the most important molecular techniques applied to detection and differentiation of avian mycoplasmas will be presented.

ESTIMATION OF G+C RATIO AND PLASMID IDENTIFICATION

Estimation of G+C ratio in the genomic DNA is one of the oldest techniques for rough characterization of DNA of an organism. Until recently, this was also the only officially required DNA analysis for the description of new mycoplasma species (Razin and Freundt, 1984). The method is based on the estimation of relative proportion of G+C nucleotide pairs in the total genomic DNA using different techniques (melting point estimation, density gradient centrifugation). Mycoplasmas in general are characterized by very low G+C ratios (even below 30%). The method requires a large amount of high quality genomic DNA which can be isolated only from cultured mycoplasma. In addition the method requires special equipment, which is expensive, and is not part of the standard equipment in the microbiology laboratories. The results enable rough taxonomic positioning of the organism but are of no diagnostic value.

Appearance of plasmids in mycoplasmas is limited to a few strains and therefore their presence is an information of certain diagnostic value. Due to very low frequency of plasmids in

mycoplasma and the possibility that different mycoplasma strains might share the same plasmid is identification of plasmids in mycoplasmas only an accessory step in the characterization of mycoplasmas and less important for diagnostic procedures. Inspite of the fact that no plasmids have been detected in avian *Mycoplasma* species to date, the plasmids offer the possibility for labelling of genetically modified vaccinal strains

RESTRICTION ENZYME ANALYSIS OF GENOMIC DNA

Due to the small genome size of mycoplasmas ranging from 600 to 1,600 kb (Morowitz and Wallace, 1973), the restriction enzyme analysis (REA) is a very informative method for differentiation among mycoplasma strains of a particular species. The method requires relatively large amounts of high quality genomic DNA which can only be obtained from the mycoplasma culture. Wide range of restriction enzymes can produce rather complex but distinct restriction patterns of genomic DNA from different strains of certain mycoplasma species. Some enzymes are sensitive to inpurities in the DNA (proteins, traces of phenol, high concentration of salts) which can reduce the enzyme activity or even cause unspecific cleavage. The high quality of the genomic DNA is therefore necessary for reproducible results. Differentiation of related strains of *Mycoplasma gallisepticum* (Kleven *et al*., 1988) and *Mycoplasma synoviae* (Morrow *et al*., 1990) has been demonstrated using a number of restriction enzymes. The most commonly used enzymes in this type of studies are *Eco*RI, *Hin*dIII, *Bam*HI, *Bgl*II, *Kpn*I, *Xho*I, *Pst*I and *Hin*fI. In *Mycoplasma gallinarum* the specific restriction patterns of different strains were used for epizootiological study revealing very subtile differences between related strains (Dovč *et al*., 1991). Additional resolution can be achieved by using advanced electrophoretic techniques for separation of DNA fragments. Beside the standard high quality agarose gel electrophoresis the pulse field- or inversed field gel electrophoresis can be used. The strength of the method is analysis of the whole genomic DNA and highly informative results for differentiation among related strains. The disadvantages, however, are the requirement for large amounts of high quality genomic DNA, time consuming procedure, difficult standardization of electrophoretic results among runs and among different laboratories and relative complexity of electrophoretic patterns which requires some experience in the interpretation of results.

HYBRIDIZATION TECHNIQUES

The method is based on the recognition of target regions in the mycoplasma genome by the labelled DNA probes. The selection of an appropriate DNA probe is of crucial importance in diagnostic procedures. Defined fragments of genomic DNA with known function can be used as hybridization probes. The most commonly used DNA probes for *Mycoplasma gallisepticum* represent fragments from rRNA genes (Razin, 1985), elongation factor Tu (Yogev *et al*., 1988, Henrich *et al*., 1996), cytadhesin (Dohms *et al*., 1993) and coding regions for a major adhesin pMGA (Baseggio *et al*., 1996). In some cases the anonymous sequences can also reveal genomic polymorphysms (Zhao and Yamamoto, 1993, Dovč *et al*., 1994). The traditional labelling of DNA probes included use of radioactive substances (^{32}P, ^{35}S, ^{3}H). The use of nonradioactive labels (biotin, digoxigenin, fluorochromes) and enzymes (alkaline phosphatase or horseradish peroxidase bound via amino link) for the labelling of DNA probes is more and more popular. Different DNA labelling has little or no influence on the assay sensitivity, therefore for the safety reasons the clear advantage is on the side of nonradioactive labelling. DNA hybridization in combination with the restriction analysis (Southern blot) is time consuming and can be replaced in some applications by dot blot procedure. The additional improvement of the method is

transformation of the dot blot assay in the microtiter plate formate and photometric quantification of hybridization results. In some cases this type of the test allows direct application of clinical material without prior cultivation of mycoplasmas. The hybridisation of RNA isolates from mycoplasmas (Northern blot) allows the expression studies and estimation of transcriptional activity at different loci.

POLYMERASE CHAIN REACTION (PCR)

Discovery of thermo stable DNA polymerases (*Taq*, *Tth*, *Pfu* and others) and design of cyclic amplification of defined genomic fragments using short synthetic oligonucleotides (Saiki *et al*., 1985) revolutionized the entire analysis of DNA. Introduction of PCR enabled in vitro amplification of defined genomic regions directly from clinical specimen without previous cultivation. Tracheal swabs and synovial fluid can be directly used as a source of DNA for amplification. Due to the relatively short length of the amplified region (normally from several hundred bp to one kb) also partially degraded template DNA can be used as a template. The region of interest is encompassed by synthetic oligonucleotides which are used as primers for *Taq* polymerase. For diagnostic purposes different regions of the genome can be used as a template for *in vitro* amplification. The most frequently *in vitro* amplified regions are fragments of 16S rRNA genes, 16S/23S ribosomal intergenic spacer region, coding regions for membrane proteins, evolutionary conserved proteins and anonymous polymorphic regions. PCR is also useful for identification of closely related species as *Mycoplasma gallisepticum* and *Mycoplasma imitans* (Bradbury *et al*., 1993). A special PCR application relies on the use of short random oligonucleotides which allow random amplification of polymorphic DNA fragments (RAPD). The result are numerous polymorphic bands which can be used as molecular markers for strain and species differentiation. Amplified regions can be further analysed by restriction enzymes and electrophoresis. Small differences in the nucleotide sequence can be determined by denaturing gradient gel electrophoresis (DGGE) or electrophoresis of single strand DNA under native conditions (Single Strand Conformation Polymorphism, SSCP). The combination of reverse transcription (RT) and subsequent PCR allows amplification of RNA molecules and identification of viable cells. In some assays a two step PCR, which is based on the use of the second pair of internal primers (nested PCR), can increase specificity of the test. Theoretically one single copy of the mycoplasma genome is sufficient for *in vitro* amplification of the target region. This incredible sensitivity might cause some confusion in diagnostic work. From the clinical point of view, the detection of DNA of one or a very low number of cells of mycoplasmas does not prove infection. Therefore several positive and negative controls should be included in every diagnostic PCR. In relation to frequent amplification of short DNA fragments, the possibility of contamination of the PCR reaction with the products from the previous reactions (which are effective templates) is a serious hazard to every diagnostic laboratory. In practice different strategies are used to prevent uncontrolled carry over of the PCR products: special isolation of different working steps, use of special pipetting techniques and use of dUTP instead of dTTP in order to allow degradation of old products by UNG-glycosylase prior to every PCR run.

PCR has frequently been used for the detection of the pathogenic avian mycoplasmas in infected flocks and numerous PCR based tests were published (reviewed in Kempf, 1997). Different target regions, including anonymous sequences (Nascimento *et al*., 1991, Dovč *et al*., 1992) and rRNA genes (Kempf *et al*., 1995) were used. For the diagnostic purposes is very informative amplification of the rRNA gene using semi conserved primers (amplifying rRNA genes of *M. gallisepticum*, *M. synoviae* and *M. iowae*) following by the restriction analysis in order to identify each of the three species (Garcia *et al*., 1996). Some approaches enabled

efficient differentiation of mycoplasma species based on restriction analysis of larger amplified regions (Fan *et al*., 1995a). This method is limited to identification of mycoplasma DNA from pure cultures because otherwise other bacterial DNA might be amplified as well. The use of short arbitrary primers (RAPD) enabled differentiation among strains of *M. gallisepticum*, *M. synoviae*, *M. iowae* and *M. meleagridis* (Fan *et al*., 1995b).

DNA SEQUENCING

In spite of the high resolution power of the methods described already, the final information about the structure of the genome can be obtained only by DNA sequencing. The development of new sequencing kits and automated DNA sequencers enabled realization of large genomic projects with the final goal to obtain the DNA sequence of the entire organism. Mycoplasmas are suitable object for such projects due to small size of their genome. Two mycoplasma genomes have already been fully sequenced (*M. genitalium, M. pneumoniae*) and the post genomic research on genome function can begin (Fraser *et al*., 1995, Himmelreich *et al*., 1997). Beside the global genomic analysis the variation of several genomic regions can reveal useful differences among strains and contribute to the sequence based diagnostic procedures, which are especially appropriate in the case of highly variable genomic regions such as 16S rRNA genes (Johansson *et al*., 1996) and the major adhesin gene *pMGA* in *M. gallisepticum* (Dovč *et al*., 1998). The special importance of sequence data can be demonstrated in phylogenetic studies where the detailed information about very variable parts of the genome (Kimura, 1980) can significantly improve the resolution of phylogenetic trees.

SUMMARY

Due to problems associated with the growth of fastidious mycoplasmas the introduction of DNA based diagnostic methods is of great importance. Molecular methods enable analysis of the genetic blue print of the organisms independent on physiological status of the organism and environmental conditions. Several methods for mycoplasma identification and differentiation have been developed. The G+C molecular ratio estimation and plasmid detection are less informative and not suitable for diagnostic purposes in avian *Mycoplasma* species. The restriction enzyme analysis (REA) of the entire genomic DNA was the first technique applied to differentiation of strains of avian *Mycoplasma* species. The small size of the genome and large choice of restriction enzymes offered the possibility to produce complex but informative RFLP patterns as an useful tool for epizootiological studies. The increasing number of cloned mycoplasma genomic fragments resulted in a number of informative DNA probes which were successfully used for species identification and differentiation of strains within the species. The most commonly used probes were fragments of 16S rRNA genes, 16S/23S ribosomal intergenic spacer region, cytadhesin genes and elongation factor-Tu (EF-Tu) gene. In addition, a number of species- and strain-specific anonymous sequences was cloned and used for hybridization studies. The polymerase chain reaction (PCR) enabled identification of *Mycoplasma* species using specific short synthetic oligonucleotides without isolation of the organism and without the need to send cloned DNA probes from laboratory to laboratory. This advantage of the PCR reduced the shipping of biological material between laboratories considerably. The specificity of the reaction can be optimised to the degree that only one species or even one strain can be amplified. The combination of PCR and RFLP enabled differentiation of related strains without their isolation from the clinical material. Even the presence of few genome equivalents in the sample enabled amplification and identification of the relevant strain and might point to the source of

infection. Only short sequence information from both ends of the target DNA is required for successful amplification. PCR based tests for the most important avian *Mycoplasma* species (*M. gallisepticum, M. synoviae, M. meleagridis and M. iowae*) were established. Application of short arbitrary primers for PCR enabled amplification of several genome fragments at the same time and differentiation of mycoplasma strains without previous knowledge about genomic DNA sequence. Recent advances in genome research provided the first complete genomic sequences of mycoplasma genomes (*M. genitalium, M. pneumoniae*) containing an incredible amount of information which brought a new insight into the fine genomic structure of mycoplasma genome. The application of molecular techniques will provide better understanding of basic biological principles and allow improvement of diagnostic procedures.

POVZETEK

Zaradi težav pri kultivaciji počasi rastočih mikoplazem je uvedba molekularnih tehnik v diagnostiki mikoplazemskih infekcij bistvenega pomena. Molekularne tehnike nam omogočajo analizo dednega zapisa organizmov, neodvisno od njihovega fiziološkega stanja in delovanja okoliških faktorjev. Razvite so bile različne metode za identifikacijo in diferenciacijo mikoplazemskih infekcij, ki temeljijo na analizi DNK. Analiza restrikcijskih fragmentov, s katero lahko zajamemo celotno genomsko DNK, se je najprej uveljavila za ločevanje sevov znotraj vrst ptičjih mikoplazem. Majhen genom mikoplazem in velik izbor restrikcijskih encimov omogočata identifikacijo informativnih RFLP vzorcev, ki jih lahko uporabljamo v epizootioloških študijah. Vedno večje število kloniranih odsekov genoma mikoplazem omogoča uporabo številnih informativnih hibridizacijskih sond za identifikacijo in diferenciacijo sevov znotraj določene vrste mikoplazem. Najpogosteje uporabljane sonde so fragmenti genov za 16S rRNK, 16S/23S ribosomska intergenska regija, geni za citadhezine in gen za elongacijski faktor Tu (EF-Tu). Poleg tega so bile klonirane tudi številne anonimne genomske regije za identifikacijo vrst in sevov ptičjih mikoplazem. Polimerazna verižna reakcija (PCR) omogoča *in vitro* pomnoževanje genomskih odsekov ob uporabi kratkih sintetičnih oligonukleotidov, zaradi česar pošiljanje klonov in drugega biološkega materiala med laboratoriji ni več potrebno. Specifičnost PCR reakcije lahko optimiramo do te mere, da pomnožujemo le genomske fragmente ene same vrste ali celo enega samega seva. Kombinacija PCR in RFLP omogoča razlikovanje med podobnimi sevi brez predhodne izolacije mikoplazem iz kliničnega materiala. Celo prisotnost ene same celice ali zelo majhnega števila celic je dovolj za amplifikacijo in identifikacijo seva, kar lahko nakaže vir infekcije. Za uspešno amplifikacijo moramo poznati le kratek delček nukleotidnega zaporedja na obeh straneh tarčne regije. Uveljavljeni so PCR testi za identifikacijo različnih vrst ptičjih mikoplazem (*M. gallisepticum*, *M. synoviae*, *M. meleagridis* in *M. iowae*). Za amplifikacijo genomskih regij, na osnovi katerih lahko razlikujemo med sevi mikoplazem, ne da bi poznali nukleotidno zaporedje teh regij, uporabljamo kratke naključne oligonukleotide. Napredek pri sekvenciranju celotnih genomov mikoplazem (*M. genitalium*, *M. pneumoniae*) nam z množico informacij odpira vpogled v strukturo genoma in omogoča boljše razumevanje osnovnih bioloških principov delovanja mikoplazemske celice.

REFERENCES

Baseggio, N./ Glew, M. D./ Markham, P./ Whithear, K.G./ Browning G.F. Size and genomic location of the pMGA multigene family of *Mycoplasma gallisepticum*. Microbiology, 142(1996), 1429-1435.

Benčina, D./ Bradbury, J. M. Combination of Immunofluorescence and Immunoperoxidase Techniques for Serotyping Mixtures of Mycoplasma Species. J. Cli. Microbiol., 30(1992), 407-410.

Bradbury, J. M./ Abdul-Wahab, O. M. S./ Yavari, C. A./ Dupiellet, J. P./ Bove, J. M. *Mycoplasma imitans* sp. Nov. is related to *Mycoplasma gallisepticum* and found in birds. Int. J. Syst. Bacteriol., 43(1993), 721-728.

Dohms, J. E./ Hnatow, P./ Morgan, R./ Keeler, C. L.. Identification of the putative cytadhesin gene or *Mycoplasma gallisepticum* and its use as a DNA probe. Avian Dis., 37(1993), 380-388.

Dovč P./ Benčina D./ Miločevič Berlič, T./ Drobnič-Valič, M./ Narat, M. Localized changes in *Mycoplasma gallisepticum* PMGA gene associated with antigenic variants. Proceedings of the 12th IOM Conference 22-28 July, 1998 Sydney, Australia, D.34, 137-138.

Dovč, P./ Benčina, D./ Zajc, I. Genotypic heterogeneity among strains of *Mycoplasma gallinarum*. Avian Pathology, 20(1991), 705-711.

Dovč, P./ Benčina, D./ Antes, R./ Mann, W. Recombinant DNA probes and polymerase chain reaction for detection of *Mycoplasma gallisepticum* strains. FEMS Microbiology, Letters, 122(1994), 79-84.

Fan, H. H./ Kleven, S. H./ Jackwood, M. W. Application of polymerase chain reaction with arbitrary primers to strain identification of *Mycoplasma gallisepticum*. Avian Dis., 39(1995a), 729-738.

Fan, J. H./ Kleven, S. H./ Jackwood, M. W. Studies of intraspecies heterogeneity of *Mycoplasma synoviae*, *M.meleagridis*, and *M.iowae* with arbitrarily primed polymerase chain reaction. Avian Dis., 39(1995b), 766-777.

Fraser, C. M./ Gocayne, J. D./ White, O./ Adams, M. D./ Clayton, R. A./ Fleischamann, R. D./ Bult, C. J./ Kerlevage, A. R./ Sutton, G./ Kelley, J. M./ Fritcherman, J. L./ Weidman, J. F./ Small, K. V./ Sandusky, M./ Furhmann, J./ Nguyen, D./ Utterback, T. R./ Saudek, D. M./ Phillips, C. A./ Merrick, J. M./ Tomb, J./ Dougherty, B. A./ Bott, K. F./ Hu, P./ Lucier, T. S./ Peterson, S. N./ Smith, H. O./ Hutchiston III, C. A./ Venter, J. C.. The minimal gene complement of *Mycoplasma genitalium*. Science, 270(1995), 397-403.

Garcia, M./ Jakwood, M. W./ Head, M./ Levisohn, S./ Kleven, S. H. Use of species-specific oligonucleotide probes to detect *Mycoplasma gallisepticum*, *M.synoviae*, and *M.iowae* PCR amplification products. J. Vet. Diagn. Invest., 8(1996b), 56-63.

Henrich, B/ Kamla, V./ Hadding, U. Phylogeny based on elongation factor Tu better reflects phenotypic features of the smallest living organisms. IOM, Letters, 4(1996), 294-95.

Himmelreich R./ Plagens H./ Hilbert H./ Reiner B./ Herrmann R. Comparative analysis of the genomes of the bacteria *Mycoplasma pneumoniae* and *Mycoplasma genitalium*. Nucleic Acids Res., 25(1997), 701-712.

Johansson, K. E./ Boelske, G./ Petterson, B. Polymorphism and sequence lenght variation in the 16S rRNA genes of some ruminant mycoplasmas. IOM, Letters, 4(1996), 292-293.

Kempf, I. DNA amplification methods for diagnosis and epidemiological investigations of avian mycoplasmosis. Acta Veterinaria Hungarica, 45(1997), 373-386.

Kimura, M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol., 16(1980), 111-120

Kleven, S. H./ Browning, G. F./ Bulch, D. M./ Ghioca, E./ Morrow, C. J./ Whithear, K. G. Examination of *Mycoplasma gallisepticum* strains using restriction endonucleases DNA analysis and DNA-DNA hybridization. Avian Pathol., 17(1988), 559-578.

Morowitz H. J./ Wallace D. C. Genome size and life cycle of the Mycoplasma. Ann N Y Acad Sci., 225(1973), 62-73.

Razin, S. DNA probes and PCR in diagnosis of mycoplasma infections. Mol. Cell. Probes, 8(1994), 497-511.

Razin, S. Molecular approach to mycoplasma phylogeny. In: Whitcomb, R. F./ Tully, J. G. (eds.). The Mycoplasmas, Vol. 5, New York, Academic Press, 1985, 33-69.

Razin, S./ Freundt, E. A. The mycoplasmas In: Krieg, N. R./ Holt, J. G. (eds.). Bergey's Mannual of Systematic Bacteriology, Vol. 1, Baltimore, The Williams&Wilkins Co, 1984, 740-793.

Saiki, R. K/ Scharf, S./ Faloona, F./ Mullis, K. B./ Horn, G. T./ Erlich, H. A./ Ahnheim, N. Enzymatic amplificato of globine sequences and restriction site analysis for diagnosis of sickle cell anemia. Science, 230(1985), 1350-1354.

Su, C. J./ Chavoya, A./ Baseman, J. B. Regions of *Mycoplasma pneumoniae* cytadhesin P1 structural gene exist as multiple copies. Infect Immun, 56(1988), 3157-61.

Yogev, D./ Sela, S./ Bercovier, H./ Razin, S. Elongation factor (EF-Tu) gene probe detects polymorphism in *Mycoplasma* strains. FEMS Microbiol., Letters, 50(1988), 145-149.