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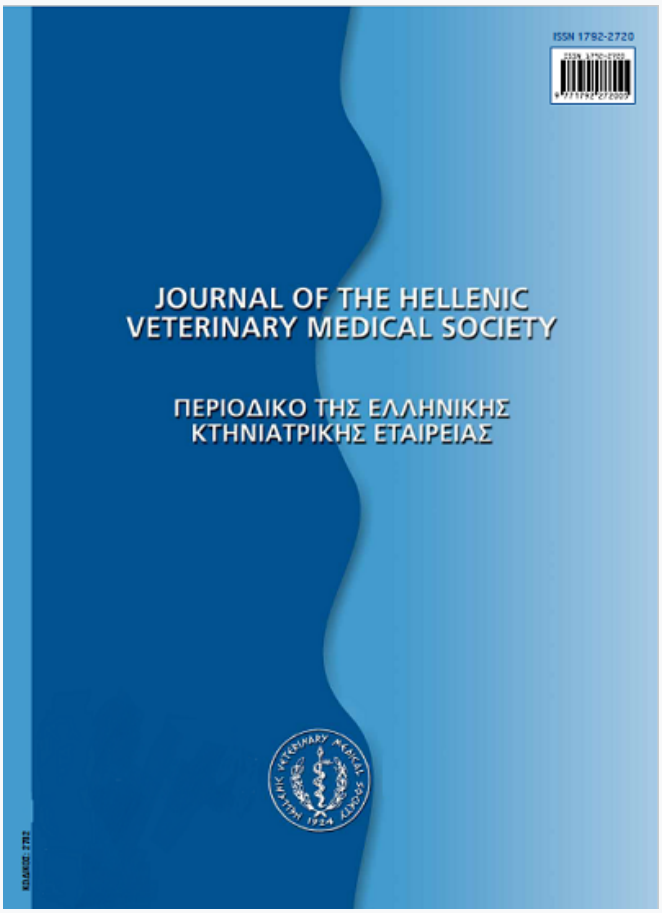

Differentiation of leptospiral serovars by restriction endonucleases analysis

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ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ 2006, 57(4): 297-301
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Ερευνητική
Original article

Διαφοροποίηση των στελεχών λεπτόσπειρας με περιοριστικά ένζυμα

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ΠΕΡΙΛΗΨΗ.* DNA από δέκα στελέχη λεπτόσπειρας χρησιμοποιήθηκε για πέψη με τα ένζυμα περιορισμού *Hind*III, *BamH*I και *EcoR*I, με σκοπό τη διαφοροποίησή τους ανά ορότυπο. Μεταξύ των ορότυπων που εξετάστηκαν διαπιστώθηκε υψηλή ετερογένεια. Ομοιότητες αναδείχθηκαν μεταξύ στελεχών του ίδιου ορότυπου. Δεν διαπιστώθηκε συσχέτιση μεταξύ του είδους από το οποίο απομονώθηκαν τα στελέχη που εξετάστηκαν και των τύπων στους οποίους διακρίθηκαν βάσει τυποποίησης με ένζυμα περιορισμού. Φαίνεται ότι η ανάλυση με τη χρήση ενζύμων περιορισμού θα ήταν χρήσιμη στη διάκριση ορότυπων της λεπτόσπειρας.

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Differentiation of leptospiral serovars by restriction endonucleases analysis

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ABSTRACT. DNA from ten leptospiral field isolates was digested with restriction enzymes, *Hind*III, *BamH*I and *EcoR*I to differentiate leptospiral serovars. A high heterogeneity among the serovars examined was revealed. Similarities were observed among isolates of the same serovar. No relationship was found between the restriction patterns and the species from which the field strain was isolated. It appears that restriction endonucleases analysis would be useful in the differentiation of the leptospiral serovars.

Key words: Leptospira, serovars, restriction enzymes

INTRODUCTION

Leptospirosis is a significant zoonotic disease with important veterinary and public health impact. Different leptospira species cause various forms of the disease in man and animals that are collectively referred to as leptospirosis. Leptospires are members of the *Leptospiraceae* family within the order *Spirochaetales* (Garrity and Holt, 2001). The genus *Leptospira* was formerly divided into two species: *Leptospira interrogans* that is the pathogenic species and *Leptospira biflexa* that is the free-living saprophyte. Both *L. interrogans* and *L. biflexa* are divided into numerous serovars. Over 60 serovars of *L. biflexa* and more than 200 serovars of *L. interrogans* are recognized. Serovars that are antigenically related have traditionally been grouped into serogroups. The phenotypic classification of leptospires has been replaced by a genotypic one that comprises of approximately 16 species for the entire *Leptospira* spp (Yasuda et al., 1987; Ramadass et al., 1992; Perolat et al., 1998; Brenner et al., 1999; Levett, 2001). The genetically defined species of *Leptospira* do not correspond to the previous two species and both pathogenic and non-pathogenic serovars occur within some species. Thus, neither serogroup nor serovar reliably predicts the species of *Leptospira*. Genetic heterogeneity within serovars occurs resulting in strains of some serovars being classified in multiple species (Brenner et al., 1999; Feresu et al., 1999; Levett, 2001).

The identification of leptospiral serovars was performed by the cross agglutination absorption test, but this method did not differentiate strains within serovars (Hathaway et al., 1985). DNA-based techniques were introduced to identify leptospiral serovars and even they couldn't differentiate between field strains (Zuerner and Bolin, 1990; Pacciarini et al., 1992; Djordjevic et al.,1993; Zuerner et al., 1993; Corney and Colley, 1996; Bolin and Zuerner, 1996; Letocard et al., 1997; Rocha, 2004). Restriction endonuclease analysis (REA) has been widely used as an epidemiological tool and a typing method for bacterial isolates of public

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Table 1. Leptospiral field isolates and reference strains used in the study

Animal species	Origin	Serovar	Name
Human	USA	*portland-vere*	Ca-12-002
Dog			Ca-12-005
Pig	USA	*copenhageni*	Ic-02-003
Cattle			Ic-02-004
Cattle	USA	*Hardjo*	HB-15B-012
Cattle			HB-15B-013
Cattle	USA	*Grippotyphosa*	Gr-01-002
Pig			RM211
Pig	USA	*kennewicki*	RM211
Pig			Po-06-013
Reference strain	WHO Leptospira Reference Lab. (Australia)	*Canicola*	Ca-re
Reference strain	WHO Leptospira Reference Lab. (Australia)	*Hardjo (strain hardjoprajitno)*	HB-re

health or veterinary importance. Marshall et al. (1981) were the first to apply the REA in the classification of leptospires. Since then, after the improvement of DNA extraction methods and the resolution of the restriction fragments, the REA has been widely used to differentiate and classify leptospires (Marshall et al., 1984; Thiermann et al., 1985, 1986; Terpstra et al., 1987; Tamai et al., 1988; Ellis et al., 1988; Thiermann and Le Vebvre, 1988; Silbreck and Davis, 1989; Zuerner and Bolin., 1990; Woodward and Redstone, 1993; Corney and Colley, 1996; Bolin and Zurner, 1996). The technique has become more sensitive and an accurate taxonomic tool and hence genotyping differences were revealed among field isolates and their corresponding reference strains, whereas cross agglutination absorption test has failed to detect these differences.

The main objective of the present study was to study and compare the DNA profiles of leptospiral field isolates by restriction endonuclease analysis.

MATERIALS AND METHODS

Leptospiral Isolates

Ten field isolates (belong to 5 serovars) and two reference strains were used in this study. The field isolates and reference strains details are shown in Table 1. The field isolates were isolated from clinical incidents of leptospirosis. The isolates were identified serologically based on microscopic agglutination test.

Preparation of Chromosomal Leptospiral DNA

The field isolates and reference strains were cultured in Johnson-Seiter (JS) medium. Then the cultures were incubated for up to 10 days at 30°C. Genomic DNA was extracted from the leptospiral reference strains and field isolates cultures using a commercial genomic DNA purification kit (Wizard R, Promega, USA) that was used based on the recommendations of the manufacturer.

Digestion with Restriction Endonucleases

The restriction endonucleases used for digestion were *Hind*III, *BamH*I and *EcoR*I (Promega, USA). The enzymes were selected according to Ellis et al., 1991. The digestion reaction mixture used, comprised of the DNA sample, reaction buffer, enzyme and distilled water. A total digestion mixture of 20 μl was used. One to three microgram of purified leptospiral DNA was digested with 4 to 5 U of restriction enzyme. Digestion was done at 37°C for 1 hour in the recommended buffer.

Agarose Gel Electrophoresis of Digested Products

The DNA fragments, resulting from the digestion with restriction enzymes, were separated by means of electrophoresis in 0.7% agarose in tris-borate buffer

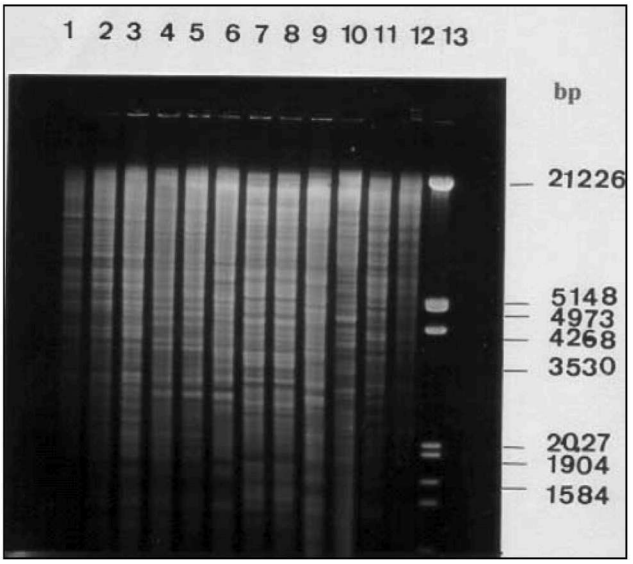

Figure 1. REA Profiles of Leptospiral Field Isolates after Digestion with *Hind*III and Electrophoresed on 0.7% Agarose Gel. Lanes: 1, *Canicola* reference strain; 2, Ca-12-002; 3, Ca-12-005; 4, Ic-02- 003; 5, Ic-02-004; 6, *Hardjo* reference strain; 7, HB-15B-012; 8, HB-15B-013; 9, RM 52; 10, Gr-01-002; 11, RM 211; 12, Po-06-013, 13, Molecular size marker.

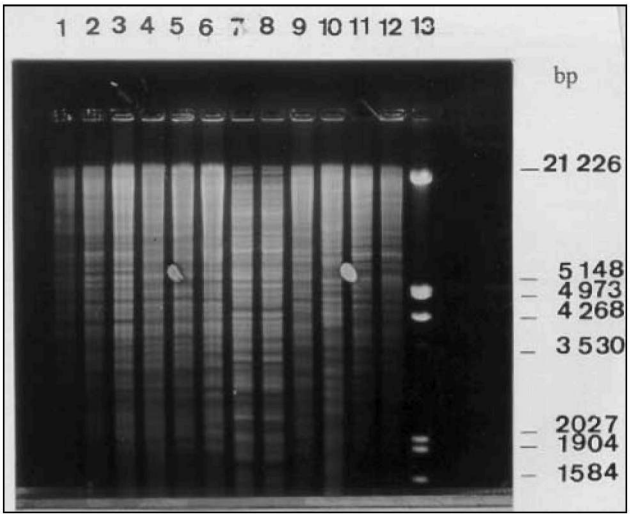

Figure 2. REA Profiles of Leptospiral Field Isolates after Digestion with *BamH*I and Electrophoresed on 0.7% Agarose Gel. Lanes: 1, *Canicola* reference strain; 2, Ca-12-002; 3, Ca-12-005; 4, Ic-02- 003; 5, Ic-02-004; 6, *Hardjo* reference strain; 7, HB-15B-012; 8, HB-15B-013; 9, RM 52; 10, Gr-01-002; 11, RM 211; 12, Po-06-013, 13, Molecular size marker.

(0.1 M Tris - 0.089 M boric acid - 0.002 M EDTA, pH 8.4) at 60 V for 18 hours. The gel was stained in ethidum bromide (0.5 μg /ml) and was photographed under UV light.

RESULTS

The restriction patterns produced by digestion of the leptospiral field isolates DNA with *Hind*III, *BamH*I and *EcoR*I are presented in Figures 1, 2 and 3, respectively. Generally, the digestion with the three enzymes separately produced a high heterogeneity among serovars, while similarities were observed among the isolates of the same serovar. Particularly the two isolates of the serovar *portland-vere produced* identical restriction patterns by each of the three enzymes, as did the isolates of serovars, *hardjo* and *kennewicki*. The two isolates of serovar copenhageni produced identical restriction patterns with H*ind*III and *BamH*I, while one isolate of this serovar (Ic-02-003) was not digested with *EcoR*I. Although the two isolates of serovar grippotyphosa were identified serologically, they seem to be different by restriction patterns by each of the three enzymes. Although the two isolates HB-15B-012, HB-15B-013 were identical and were identified serologically as Leptospira hardjo, they were quite different from *Leptospira hardjo* strain *hardjoprajitno* in their DNA profile with each of the three enzymes. The *canicola* reference strain produced a restriction pattern identical

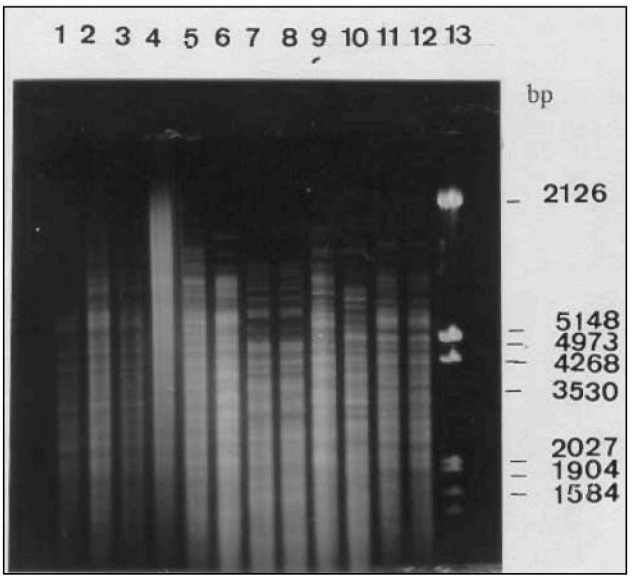

Figure 3. REA Profiles of Leptospiral Field Isolates after Digestion with *EcoR*I and Electrophoresed on 0.7% Agarose Gel. Lanes: 1, *Canicola* reference strain; 2, Ca-12-002; 3, Ca-12-005; 4, Ic-02- 003; 5, Ic-02-004; 6, *Hardjo* reference strain; 7, HB-15B-012; 8, HB-15B-013; 9, RM 52; 10, Gr-01-002; 11, RM 211; 12, Po-06-013, 13, Molecular size marker.

to that shown by the two isolates of serovar *portland-vere*. Knowing that the two serovars belong to serogroup canicola.

DISCUSSION

After detection of leptospiral infection, it is often necessary for diagnostic and epidemiological purposes to identify the serovar involved (Savio et al., 1994). In the present study, a comparison between DNA profiles of leptospiral field isolates was examined based on restriction endonuclease analysis (REA). At the same time the relationship between the digestion pattern, the serovar and origin of the isolates was assessed. The digestion with enzymes *Hind*III, *BamH*I and *Eco*RI revealed a high heterogeneity between the serovars examined. The most interesting result in this study was the observation of no common fragment shared between all the serovars with any of the enzymes used. The high heterogeneity among leptospiral serovars would support the concept of the serovar as the basic taxonomic unit of leptospiral classification (Brown and Levett, 1997). The variable restriction patterns observed in this study will be useful in the differentiation and classification of leptospiral serovars. The identical restriction patterns of the two isolates of one serovar would suggest a common source or close genetic correlation or that the isolates were belonging to the same strain, whereas the markedly dissimilar patterns would indicate otherwise. In the present study, an identical restriction pattern was observed among the isolates of the same serovar using the three enzymes *Hind*III, *BamH*I and *Eco*RI. So the two isolates from *serovars portland-vere, copenhageni, hardjo* and *kennewicki* produced identical restriction patterns. Shi et al . (2000) study digestion by *Eco*RI of 12 field strains of *Leptospira interrogans* serovar *hebdomadis* and *australia*. The same serovar field strains of *Leptospira interrogans* resulted in unique restriction endonuclease patterns. The chromosomal DNAs from field strains that belonged to different serovars of *Leptospira interrogans* produced different restriction endonuclease patterns. One of the important findings in this study was the absence of the correlation between the restriction pattern and the species from which the isolate originated. The two isolates of serovar *portland-vere* were cultured from different host species, but produced identical restriction patterns with all three enzymes. The same was observed in the isolates of serovar Copenhagen. The two isolates of serovar *hardjo* originated from same animal species and produced identical restriction patterns. These findings support previous reporteds about no detectable variation in the restriction endonuclease fingerprints among isolates from different species of animals or among isolates from the same host species (Marshall et al., 1981; Robinson et al., 1982, Bolin and Zuerner, 1996; Djorjevic et al., 1993). Identical restriction patterns of isolates from different species was suggestive of the stability of the genome of the organism. The field strains of *hardjo* had, however, a completely different fragment pattern from that of the *hardjo* reference strain *hardjoprajitno*. Heterogeneity among isolates belonging to same serovar (subserovar, subtype) has been shown by Thiermann et al. (1985, 1986); Ellis et al. (1991); Bolin and Zuerner (1996); Khairani (1997); Boqvist et al., 2003). In conclusion, high heterogeneity was revealed between the serovars examined using REA, while similarities were observed among the isolates of same serovar, regarding the serovars portland-vere (canicola), Copenhagen and Kennewicki. ❑

ΒΙΒΛΙΟΓΡΑΦΙΑ - REFERENCES

Bolin, C. A and Zuerner, R. L. (1996). Correlation between DNA restriction fragment length polymorphisms in Leptospira interrogans serovar pomona type kennewicki and host animal source. J Clin Microbiol. 34:424-425.

Boqvist, S., Montgomery, JM., Hurst, M., Thu, H.T., Engvall, E.O., Gunnarsson, A and Magnusson, U.(2003). Leptospira in slaughtered fattening pigs in southern Vietnam: presence of the bacteria in the kidneys and association with morphological findings. Vet Microbiol. 93:361-368.

Brenner, D. J., Kaufmann, A. F., Sulzer, K. R., Steigerwalt, A. G., Rogers, F. C and Weyant, R. S. (1999). Further determination of DNA relatedness between serogroups and serovars in the family Leptospiraceae with a proposal for Leptospira alexanderi sp. nov. and four new Leptospira genomospecies. Int J Syst Bacteriol. 49 :839-858.

Brown, P. D. and Levett, P. N. (1997). Differentiation of leptospira species and serovars by PCR, restriction endonuclease analysis, arbitrary primed PCR and low stringency PCR. Journal of Medical Microbiology. 46: 173-181.

Corney, B. G. and Colley, J. (1996). Restriction fragment length polymorphism in Australian and New Zealand isolates of Leptospira interrogans serovar pomona. Australian Veterinary Journal. 74: 156-157.

Djordjevic, S., Hornitzky, M., Ross, A. D. and Whittington, R. J. (1993). Restriction-endonuclease analysis of Australian isolates of Leptospira interrogans serovar hardjo from cattle with agalactia and abortion. Australian Veterinary Journal. 70: 98-100.

Ellis, W.A., Thiermann, A.B., Montgomery, J., Handsaker, A., Winter, P.J. and Marshall, R.B. (1988). Restriction endonuclease analysis of Leptospira interrogansserovar hardjo isolates from cattle. Res Vet Sci.443:375-379.

Ellis, W.A.,Montgomery, J.M. and Thiermann, A.B. (1991). Restriction endonuclease analysis as a taxonomic tool in the study of pig isolates belonging to the Australis serogroup of Leptospira interrogans. J Clin Microbiol. 29:957-961.

Feresu, S. B., Steigerwalt, A. G. and Brenner, D. J. (1999). DNA relatedness of Leptospira strains isolated from beef cattle in Zimbabwe. Int J Syst Bacteriol. 49:1111-1117.

Garrity, G.M. and Holt, J.G. (2001). The road map to the manual. Bergey's Manual of Systematic Bacteriology. 2nd edition. Edited by: Boone and Castenholz RW. Berlin, pringer-Verlag.

Hathaway, S.C., Marshall, R.B., Little, T. W.A., Headlam, S.A. and Winter, P.J. (1985). Differentiation of reference strains of leptospires of the Pomona serogroup by cross-agglutination and restriction endonuclease analysis. Research in Veterinary Science. 39: 145-150.

Khairani, B. (1997). A study of the leptospiral isolates obtained from cattle in Malaysia. Master Thesis, University Pertanian Malaysia.

Letocard, M., Baranton, G. and Perolat, P. (1997). Rapid identification of pathogenic Leptospira species (Leptospira interrogans, L. borgpetersenii and L. kirschneri) with species-specific DNA probe produced by arbitrary primed PCR. Journal of Clinical Microbiology. 35: 248-253.

Levett, P.N.(2001). Leptospirosis. Clin Microbiol Rev. 14:296-326.

Marshall, R.B., Wilton, B.E. and Robinson, A.J. (1981). Identification of Leptospira serovars by restriction endonuclease analysis. Journal of Medical Microbiology. 14: 163-166.

Marshall, R.B.,Winter, P.J. and Yanagawa, R. (1984). Restriction endonuclease DNA analysis of Leptospira interrogans serovars icterohaemorrhagiae and hebdomadis. J Clin Microbiol. 20:808-810.

Pacciarini, M.L., Savio, M.L., Tagliabue, S. and Rossi, C (1992). Repetitive sequences cloned from Leptospira interrogans serovar harjdo genotype hardjoprajitno and their application to serovar identification. Journal of Clinical Microbiology. 30: 1243-1249.

Perolat, P., Chappel, R.J., Adler, B., Baranton, G., Bulach, D.M., Billinghurst, M.L., Letocart, M., Merien, F. and Serrano, M.S. (1998). Leptospira fainei sp. nov., isolated from pigs in Australia. Int J Syst Bacteriol.. 48 :851-858.

Ramadass, P., Jarvis, B.D., Corner, R. J., Penny, D. and Marshall, R. B. (1992). Genetic characterization of pathogenic Leptospira species by DNA hybridization. Int J Syst Bacteriol. 42: 215-219.

Robinson, A.J., Ramadass, P., Lee, A. and Marshall, R.B. (1982). Differentiation of subtypes within Leptospira interrogans serovars hardjo, balcanica and tarassovi by bacterial restriction endonuclease analysis (BRENDA). Journal of Medical Microbiology. 15: 331-338.

Rocha, T., Ellis, W.A., Montgomery, J., Gilmore, C., Regalla, J. and Brem, S. (2004). Microbiological and serological study of leptospirosis in horses at slaughter: first isolations. Research Veterinary Science. 76:199-202.

Savio, M.L., Rossi, C., Fusi, P., Tagliabue, S. and Pacciarini, M.L. (1994). Detection and identification of Leptospira interrogans serovars by PCR coupled with restriction endonuclease analysis of amplified DNA. Journal of Clinical Microbiology.32: 935-941.

Shi, M., Liang, Z., Du, X., Jiang, X. and Nie, Y. (2000). A molecular epidemiological investigation on Leptospira interrogans serovar hebdomadis and australia in China. Zhonghua Liu Xing Bing Xue Za Zhi. 21:91-93.

Silbreck, N. W. and Davis, W. D. (1989). Restriction endonuclease analysis of Australian isolates of Leptospira interrogans serovar hardjo. Australian Veterinary Journal. 66: 183-184.

Tamai, T., Sada, E. and Kobayashi, Y. (1988). Restriction endonuclease DNA analysis of Leptospira interrogans serovars Icterohaemorrhagiae and Copenhageni. Microbiol Immunol.; 32(9):887-94.

Terpstra, W.J., Korver, H., Schoone, G.J., von Leeuwen, J., Schonemann, C.E., de Jonge-Aglibut, S. and Kolk, A.H. (1987). Comparative classification of Leptospira serovars of the Pomona group by monoclonal antibodies and restriction-endonuclease analysis. Zentralbl Bakteriol Mikrobiol Hyg. 266:412-421.

Thiermann, A.B. and Le Vebvre, R.B. (1988). Restriction Endonuclease analysis of leptospiral pathogens from North America. Israel Journal of Veterinary Medicine. 44: 62.

Thiermann, A. B., Handsaker, A. L., Moseley, S.L. and Kingscote, B. (1985). New method for classification of leptospiral isolates belonging to serogroup pomona by restriction endonuclease analysis: serovar kennewicki. J Clin Microbiol. 21:585-587

Thiermann, A.B., Handsaker, A. L., Foley, J. W., White, F. H. and Kingscote, B. F. (1986). Reclassification of North American leptospiral isolates belonging to serogroups Mini and Sejroe by restriction endonuclease analysis. American Journal of Veterinary Research. 47: 61-66.

Woodward, M.J. and Redstone, J.S. (1993). Differentiation of Leptospira serovars by polymerase chain reaction and restriction fragment length polymorphism. Veterinary Record. 132: 325-326.

Yasuda, P.H., Steigerwalt, A.G., Sulzer, K.R., Kaufmann, A.F., Rogers, F. and Brenner, D.J. (1987). Deoxyribonucleic acid relatedness between serogroups and serovars in the family Leptospiraceae with proposals for seven new Leptospira species. Int. J. Syst. Bacteriol: 1987 - 407-415.

Zuerner, R.L. and Bolin, C.A. (1990). Nucleic acid probe characterizes leptospira interrogans serovars by restriction fragment length polymorphism. Veterinary Microbiology. 24: 355-365.

Zuerner, R.L., Ellis, W.A., Bolin, C.A. and Montgomery, J.M. (1993). Restriction fragment length polymorphisms distinguish Leptospira borgpetersenii serovar hardjo type hardjo-bovis isolates from different geographical locations. J Clin Microbiol. 31:578-83