# DESCRIPTION OF REVERSE LINE BLOTTING (RLB) TECHNIQUE AND RLB BASED STUDIES AIMED TO DETERMINE THEILERIA AND BABESIA SPECIES IN SHEEP AND GOATS IN TURKEY

*REVERSE LINE BLOTTING TEKNİĞİNİN AÇIKLANMASI VE TÜRKİYE'DE KOYUN VE KEÇİLERDE THEİLERİA VE BABESİA TÜRLERİNİN BELİRLENMESİNE YÖNELİK RLB TABANLI ÇALIŞMALAR*

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Kabul Tarihi: 08 Ocak 2016

DOI:10.5505/bsbd.2016.08860

Balıkesir Sağlık Bilimleri Dergisi
ISSN: 2146-9601
e-ISSN: 2147-2238

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**ÖZET**

Keneler tıbbi ve veteriner öneme sahip 200'den fazla patojenin naklinde rol oynamaktadırlar. Bu etkenler omurgalı konakta ve vektör kenelerde tek veya miks enfeksiyonlar şeklinde bulunmaktadır. Mikroskobik ve serolojik yöntemler tür ayrımında karşılaşılan zorluklar ve çapraz reaksiyon gibi dezavantajlara sahiptir. Bu dezavantajların giderilmesi moleküler tanı metotlarının kullanılmaya başlanması ile mümkün olabilmektedir. Son yıllarda, Reverse Line Blotting (RLB) metodu kullanılarak çoklu enfeksiyonların aynı zaman diliminde tespiti yapılabilmektedir. RLB metodu, yeni tür ve genotiplerin keşfedilmesini sağlayan moleküler bir tanı metodudur. RLB testi; cins düzeyinde PZR, türe özgü probların Biodyne-C membrana bağlanması, PZR ürünlerinin denatürasyonu, denatüre ürünlerin problar ile hibridizasyonu, streptavidin-POD + biotin kompleksinin oluşumu, bu kompleksin ışık emisyonunun gerçekleşmesi ve bunun karanlık odada radyografik film üzerine aktarılarak değerlendirilmesi olmak üzere yedi aşamadan oluşmaktadır. Bu derlemede kenelerle bulaşan hastalıkların teşhisinde RLB metodunun kullanımı hakkında detaylı bilgi verilmektedir.

**Anahtar Kelimeler:** Kene, Patojen, PZR, RLB.

**SUMMARY**

Ticks play a role in transmission of more than 200 pathogens of medical and veterinary importance. These pathogens are present in form of single or mixed infections in the vertebrate host and the vector ticks. Microscopic and serological methods have some disadvantages such as difficulties in species discrimination and cross-reaction problems. Elimination of these disadvantages has been possible by using molecular techniques. Recently reverse line blotting (RLB) method has been used for determination of multiple infections simultaneously. RLB is also useful molecular tool to discover new species and genotypes. RLB is consist of seven steps as the stages of genus level PCR, binding species-specific probes to the Biodyne-C membrane, denaturation of PCR products, hybridization of denatured products with probes, formation of streptavidin-POD + biotin complex, realization of the light emission of this complex and evaluation the result by transferring this on a radiographic film in a dark room. This review provides detailed information about the usage of RLB in the detection of tick-borne infections.

**Keywords:** Tick, pathogen, PCR, RLB.

## INTRODUCTION

Ticks have harmful effects on the host by means of sucking blood and transferring many diseases. It has been determined that ticks transmit more than 200 pathogens to wild and domestic animals and humans. The protozoan parasites in these pathogens are widespread in tropical and subtropical climates.

*Theileria* and *Babesia* species are the most important tick-borne protozoan parasites and cause clinical and subclinical infections in domestic and wild animals with high mortality and morbidity [1, 2]. Diagnosis of theileriosis and babesiosis in acute cases is based on clinical signs and microscopic examination of giemsa stained blood smears [3, 4]. Animals with theileriosis and babesiosis become carriers of the agents after infection. These carrier animals in the population are very important in the epidemiology of the diseases [5]. It has been proposed that microscopic methods are insufficient [6] for diagnosis of carrier animals, false positive and negative reactions

restrict the usage of serolojical methods in the diagnosis of carrier animals [7-9]. For these reasons, molecular diagnostic methods are needed to determine *Theileria* and *Babesia* infections in the epidemiological studies.

In recent years it has began to be used Polymerase chain reaction (PCR) and some modifications like multiplex PCR, nested PCR, touchdown PCR and reverse line blotting (RLB) for species-specific detection of organisms. RLB allows diagnosis several agents simultaneously and discovery of new species and genotypes, RLB has been used often for diagnosis of subclinical *Theileria* and *Babesia* infections [10-18].

New *Theileria* and *Babesia* species or genotypes are being discovered in sheep and goats by using molecular techniques in recent years. *Theileria* sp. OT1 and *Theileria* sp. OT3 genotypes were determined in small ruminants by RLB technique in Spain [16]. Also a different genotype named as *Theileria* sp. MK was discovered in sheep and goats in the East Anatolia Region of Turkey by RLB [19]. In China, morphologically and genetically different new *Babesia* genotypes were detected in sheep and goats by using RLB and named as *Babesia* sp. Xinjiang and *Babesia* sp. Lintan [20-23]. RLB method was developed for differentiation of four *Borrelia* species in ticks in 1995 [24]. This method has successfully used for diagnosis *Theileria* and *Babesia* species, and it's usage in the parasitological researches is increasing [12, 16, 18, 19, 25].

**RLB based studies on the detection of *Theileria* and *Babesia* species in small ruminants in Turkey**

RLB method was used for the detection of *Theileria* and *Babesia* species of sheep and goats in Kayseri province, for the first time in Turkey and *B. ovis* and *T. ovis* were detected with a rate of 2.7% and 34.2% in sheep respectively [26]. Blood samples obtained from 705 sheep and 215 goats were investigated by RLB in terms of *Theileria* and *Babesia* species in the region of Eastern Anatolia and *B. ovis* (5.43%), *T. ovis* (34.56%), *Theileria* sp. MK (1.30%) and *Theileria* sp. OT3 (0.43%) were detected. *Theileria* sp. MK genotype was first defined in this study [19]. The other studies also showed that *T. ovis* (50.55%) was the most common species in sheep and goats in this region [27, 28]. Blood samples obtained from randomly selected 200 sheep and 100 goats were investigated for the presence of *Theileria* and *Babesia* species by microscopic examination and RLB in Yeşilhisar province of Kayseri, and the results showed that the molecular prevalence of *B. ovis* and *T. ovis* were 3.7% and 37.6%, respectively [25] Blood samples taken from 421 sheep and 152 goats from Kayseri, Sivas and Yozgat provinces of central Anatolia region were examined with RLB, and *B. ovis* and *T. ovis* were determined with the rates of 2.6% and 33.9%, respectively [29]. *Theileria* and *Babesia* species were investigated by microscopy and RLB in sheep and goats in the Black Sea Region in 2010-2011. While 38 (3.37%) of 1128 blood samples were positive for *Theileria* spp. in microscopic examination, in RLB analysis *T. ovis* (28.99%), *B. ovis* (0.44%), *Theileria* sp. OT3 (2.04%) and *Theileria* sp. MK (0.62%) species or genotypes were determined [30].

**Stages of PCR based Reverse Line Blotting**

**DNA extraction:** Blood samples are defrosted and homogenized at room temperature for 10-15 seconds. DNA extractions are performed by using a commercial DNA isolation kit or the manual method. In manuel method 125 µl of blood is added to 250 µl of lysis buffer (0.32 M sucrose, 0.01 M Tris, 0.005 M $MgCl_2$, 1% Triton X-100, pH 7.5). The mixture is centrifuged at 11.600 g for 1 min. The pellet is washed three times with 250 µl lysis buffer by centrifugation. The supernatants are discarded, and the final pellets are resuspended in 100 µl of PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8), 0.1% TritonX-100, pH 8.3). Proteinase K (50 µg/ml) is added to the pellet suspension, and the mixture is incubated at 56 °C for 1 h. Finally, the samples are heated at 100 °C for 10 min [31]. Genomic DNAs are kept at -20 ºC until use.

**Polymerase chain reaction and agorose jel electrophoresis:** For the amplification of *Theileria* and *Babesia* species, one set of primers are used to amplify an approximately 360-430 bp fragment of the hypervariable V4 region of the 18S rRNA gene by using the forward [RLB-F2 (5'-GACACAGGGAGGTAGTGACAAG-3')] and the reverse [RLB-R2 (Biotin-5'-CTAAGAATTTCACCTCTGACAGT-3')] primers were described by Georges et al [32]. Touchdown PCR, a modification of conventional PCR, involves the use of annealing temperatures that are higher than the optimum temperature in each PCR cycle. The annealing temperature is adjusted to be a decrease of 2 °C in every second cycle until the 'touchdown' temperature of 57°C. This allows to the enrichment of the correct product over any non-specific product. The PCR is performed in a touchdown thermocycler in a total reaction volume of 25 µl containing PCR buffer [750 mM Tris–HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 5 mM $MgCl_2$, 125 µM deoxynucleotide triphosphates, 1.25 U Taq DNA polymerase, primers (20 pmol/µl) and template DNA. 5 µl of PCR product is visualized by UV transillumination in a 1.5% agarose gel stained with ethidium bromide after electrophoresis.

***Table 1.*** *Touchdown PCR thermal conditions*

| Process | Temparature ($C^0$) – Time (sec) | Number of cycles |
|---|---|---|
| Denaturation | 94 – 20 | |
| Hybridization | 67 – 30 | 2 |
| Annealing | 72 – 30 | |
| Denaturation | 94 – 20 | |
| Hybridization | 65 – 30 | 2 |
| Annealing | 72 – 30 | |
| Denaturation | 94 – 20 | |
| Hybridization | 63 – 30 | 2 |
| Annealing | 72 – 30 | |
| Denaturation | 94 – 30 | |
| Hybridization | 61 – 45 | 2 |
| Annealing | 72 – 45 | |
| Denaturation | 94 – 45 | |
| Hybridization | 59 – 45 | 2 |
| Annealing | 72 – 45 | |
| Denaturation | 94 – 45 | |
| Hybridization | 57 – 45 | 40 |
| Annealing | 72 –-45 | |
| Last extension | 72 – 10 min | 1 |
| Waiting | +4 $C^0$ | |

**Reverse line blotting (RLB):** Probes contain N-terminal *N*-(trifluoracetamidohexyl-cyanoethyl,*N*,*N*-diisopropyl phosphoramidite [TFA])-C6 amino linker. Biodyne C membrane is activated in 10 ml of 16% EDAC 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide for 10 min at room temperature and put inside a miniblotter after washing with demineralised water. Residual liquid on the membrane is aspirated. 150 µl of each probe which diluted to a 50 to 1200 pmol/150 ml concentration in 500 mM $NaHCO_3$ (pH 8.4) is filled into the channels of miniblotter except the first and last channels. %1 India ink diluated with 2XSSPE and 0.5% SDS is filled into the first and last slots. Then the membrane is incubated for 10 min at room temperature. Liquids in slots are aspirated after incubation. The membrane is inactivated in 100 mM NaOH for 10 min, after removing from miniblotter at room temperature. Finally, it is washed in 2X SSPE/ 0.1% SDS for 5 min at 60°C.

The membrane is washed for 5 min at room temperature with 2X SSPE / 0.15 %SDS and it is placed into the miniblotter with the slots vertical on the previously connected probes. Residual liquid on the membrane is aspirated. 20 µl of PCR product is diluated in 2X SSPE / 0.1% SDS with a total 150 µl volume and it is denatured for 10 min at 99 ˚C. Denaturated PCR products are cooled on ice immediately for not combine DNA strands again. Denatured PCR samples are filled into the slots and hybridized on a flat surface for 1 hour at 42°C. Residual PCR products on the membrane are aspirated. The membrane is washed twice in 2X SSPE / 0.5% SDS for 10 min at 52 ˚C. The membrane is incubated in 10 ml of 1:4000-diluted peroxidase-labeled streptavidin in 2X SSPE/ 0.5% SDS for 30 min at 42°C. Then the membrane is washed twice in 2X SSPE / 0.5% SDS for 10 min at 42°C and twice in 2X SSPE for 5 min at room temperature. Membrane is incubated in 10 ml of ECL detection fluid for 1 min, and then it is taken on a hard surface and is covered with acetate. The membrane is incubated under an ECL hyperfilm for 30 sec to 30 min depending on the strength of signals in a dark room. Finally, ECL hyperfilm is processed with the developer and fixer solutions. Black spots occurring in rows where PCR products and probes were crossed is evaluated as positive to the related species.

For stripping of PCR-products from membrane it is washed twice in 1% SDS for 30 min at 85°C. Finally the membrane is rinsed in 20 mM EDTA for 15 min at room temperature and stored in 20 mM EDTA at 4°C for reusing.

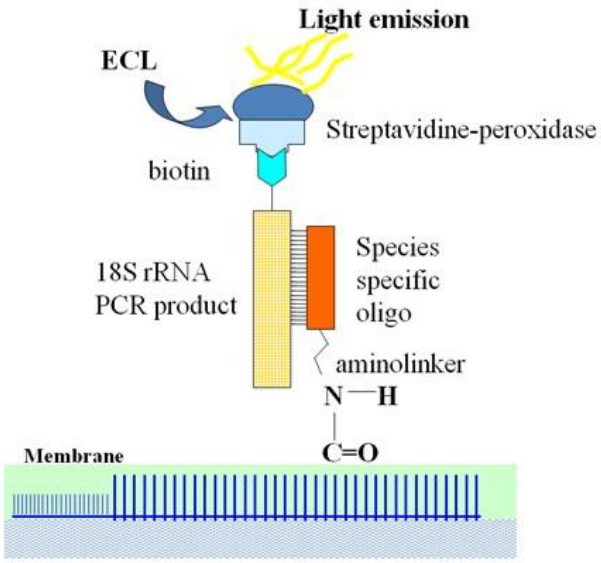


***Figure 1.*** *Principle of the Reverse Line Blotting* [33]

## CONCLUSION

In order to develop appropriate control strategies for tick borne diseases, it is required that identification of causative agents and determine the incidence of carrier animals in population. Carrier animals source of infection for tick vectors. Causative agents present in the form of single or mixed infections in host or vector tick. RLB provides simultaneous detection multiply tick-borne pathogens. So it will facilitate epidemiological studies on tick-borne diseases and leading to better control of these diseases.

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