Antigen based diagnostics: the value of PCR in crypto-infections diagnosis

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PCR from animals

Wild animals in Turkey

* *Orkrun 2018. Comp Immunol Microbiol Infect Dis*
* **Boar, hare, fox (and their ticks)**

* **PCR targets**
  * *Rickettsia spp*: gltA and ompA genes
  * *Borrelia spp*: 5S-23S rDNA gene
  * *Anaplasma spp*: msp4 gene

* **Five spotted fever group *Rickettsiae***
  * Three pathogenic and two with unknown pathogenicity
* **One *B. burgdorferi sensu lato***

* **Ticks:** 5 species of *Rickettsiae*

Metagenomic approach to analyse bacteria in rodents and ectoparasites. Thailand

* *Takhampunya 2019. Front Microbiol*

* **309 wild rodents + 420 pools of ectoparasites + 200 patients with undifferentiated febrile illness (UFI)**
* **Nan province:** highly endemic for scrub typhus (*Orientia tsutsugamushi*)

* **16S rRNA gene amplified and sequenced with Illumina**
* **Real-time PCR and Sanger sequencing**

* **In patients with UFI, rodents and parasites:** *Bartonella spp, Rickettsia spp, Leptosira spp., Orientia tsutsugamushi* (chiggers), *Anaplasma spp* (not previouly detected in humans in this area)

* **In rodents and parasites:** *Neoehrlichia mikurensis, Neorickettsia spp, Borrelia spp*

Pathogens in Eurasian Moose. Sweden

* *Malmsten 2019. Vector Borne Zoonotic Dis*
* **Spleen samples**
* **Vectors: keds** (kind of lice, *Lipoptena cervi*)
* **High-throughput PCR for 24 bacteria and 12 pathogenic parasites**
  * *Anaplasma* 82%
  * *Borrelia* 3%
  * *Babesia* 3%
  * *Bartonella* 1%

Wild animals in Slovakia and Slovenia

* **Wild ungulates in Slovakia.**
* *Kazimirova 2018. Parasit Vectors*
  * Ungulates PCR positive for:
    * *Anaplasma phagocytophilum*
    * *Theileria spp*
    * Mixed infections

* **Rodents in Slovenia**
* *Cerar 2015. BMC Vet Res.*
  * *Borrelia*: 4.3% of lung specimens and 21.7% of heart specimens (*B. afzelii*)
  * LightMix* more sensitive than a « in-house » nested PCR: 30.6% vs 16.1%
  * Rare isolation of *B. miyamotoi*

PCR from humans

PCR from skin biopsies

* **Erythema migrans**
  * *Kondrusik 2007. Ann Agric Environ Med.* Poland. 86 patients. *Borrelia* still present after 4 weeks of antibiotics
  * *Stupica 2015. PLoS One*. Austria.
    * 121 patients.
    * **PCR pos. 77.7%** vs culture pos. 55.1% (96.8% *B. afzelii*, 3.2% *B. garinii*).
    * The higher the bacterial load, the lower the response to treatment
* **Hyperkeratosis lenticularis perstans (Flegel disease)**
  * *Schwarzova 2016. Folia Microbiol (Praha)*
* **Granuloma annulare** and **Morphea**
  * *Tolkki 2018. Acta Derm Venereol*

Lyme Neuroborreliosis (LNB)

* ***Barstad 2018. J Clin Microbiol. Norway.***
  * *B. burgdorferi* antibody index in CSF: insufficient sensitivity.
  * **Real-time PCR on CSF.**
  * ***B. burgdorferi sensu lato* PCR. If positive, five singleplex for genotype determination.**
  * 58 children with LNB (28 controls).
  * Sensitivity **46%**, specificity **100%.**
  * 27/28: *B. garinii*

* **Ruzic-Sabljic 2016. Exp Rev Molecular Diagn. Review**
  * Median sensitivity from **CSF: 22.5%**

Lyme Neuroborreliosis (LNB)

* **Comparison of three real-time PCR kits.** *Maes 2017. Eur J Clin Microbiol Infect Dis*
  * **O-DiaBorburg (DIA), ISEX (GENE), Real-TM SAC**
  * **Kits analyzed on Rotorgene Q (RGQ), CFX96 and LightCycler480**
  * Good reproductibility for all assays
  * No cross-reactivity
  * The DIA kit failed to detect *B. lusitaniae*
  * Better overall performance for the GENE kit on RGQ
  * **However: failure of PCR from CSF clinical samples of LNB!**

* **Better handling of CSF.** *Forselv 2018. Infect Dis*
  * Larger CSF sample and faster handling: Trend toward better results, but not significant

PCR from synovial fluid

* *Ruzic-Sabljic 2016. Exp Rev Molecular Diagn*
* **Cases of Lyme arthritis**
* PCR more sensitive than culture
* **PCR median sensitivity**
  * US: 85%
  * Europe: 72%

Persistent *Borrelia* infection

* ***Middleveen 2018***. *Healthcare*

* Randomly selected **12 patients (already treated with antibiotics, some currently treated at time of blood sample).** Controls: 10

* **Methods:**
  * Microscopic, Histopathological, Molecular testing, Culture
  * **PCR in two independent labs (blinded) plus gene sequencing**
  * A third lab for two patients (genital secretions)

* **Culture.** Positive: 12/12
* **PCR**
  * **Blood.** **Positive: 7 / 12**
    Lab 1: 7 Lab 2: 6/7
  * **Genital secretions.** **Positive: 10 / 12**
    Lab 1: 10 Lab 2: 8/10 Lab 3: 2/10
  * **Skin.** **Positive: 2 / 12**
    Lab 1: 2 Lab 2: 1/2

Techniques of PCR

PCR. Increased number of targets

* *de Leeuw 2014. J Microbiol Meth.* The Netherlands.

* ***B. burgdorferi sensu lato*. Most frequently used PCR: plasmid encoded outer surface protein A gene (ospA) target**
  * ospA PCR doesn't detect *B. lusitaniae* & *B. miyamotoi*
  * Risk of false-negatives due to loss of plasmid

* **DNA multiplex qPCR: ospA targets plus two extra target genes** (chromosomal 5S-23S rRNA intergenic spacer & flagellin B)
  * Increased sensitivity on CSF samples (n=74 ; 15%)

Nested PCR

* *Zhang Liu Li 2015. Biomed Environ Sci*
* Universal loop-mediated isothermal amplification primers targeting the *fla* gene (*B. burgdorferi s.l.*)
* Compared with nested PCR
* 118 human sera
* Same positive rate of both methods
* Combination of both methods: more sensitive

Improved real-time PCR

* *Bil-Lula 2015. Adv Clin Exp Med.* Poland
* **Improved real-time PCR**
* 577 , including 567 **woodworkers**
* **Serology positive:**
  * Elisa IgG 33% ; Elisa IgM 21.1% ; Western blot 38.3%
* **PCR positive** (whole blood): **3.1%**

TaqMan real-time PCR

* *Nunes 2017. Ticks Tick Borne Dis*

* **1) Duplex real-time PCR targeting *fla*B of *B. burgdorferi s.l.*** & an internal control
  * High sensitivity & high specificity
  * Isolation of 20 genome equivalents (GE) of *B. burdorferi s.l.* from culture, clinical samples & ticks

* **2) Tetraplex real-time PCR targeting *fla*B gene of *B. afzelii, B. garinii, B. burgdorferi* & *B. lusitaniae***
* high specificity, but lower sensitivity
* *Isolation of 200 GE of B. afzelii, B. garinii, B. burgdorferi s.s. & B. lusitaniae*

Molecular diagnosis. Review (1) Elimination of human DNA

* *Ruzic-Sabljic 2016. Exp Rev Molecular Diagn*
* **Overwhelming ratio of human to microbial DNA**
* **Commercial kits: enrichment of microbial DNA**
  * MolYsis Basic5 kit (lyses human cells & degrades released DNA)
  * NEBNext (separates vertebrate from microbial DNA)
  * Pureprove (DNA binding protein recognizing motifs predominent in bacterial genomes)
* **MolYsis:** complete elimination of human DNA but also loss of bacterial DNA
* **Extraction.** Automatic vs manual: as effective

Molecular diagnosis. Review (2) Appropriate target

* *Ruzic-Sabljic 2016. Exp Rev Molecular Diagn*
* **Selection of an appropriate target DNA sequence** to be amplified
* **Must be genetically stable**
* **Should enable detection of all species of *B. burgdorferi sensu lato* complex**
* **16S rRNA gene, *ospA*, *fla* & *recA*:**
  * several commercial kits
  * mostly qualitative real-time PCR assays
* ***fla*: low discriminatory power** between species
* ***ospA*, *ospB* & *ospC*: located on plasmids**
  * Highly variables
  * Amplification may not occur in all strains

Molecular diagnosis. Review (3)
How to improve? Number of bacteria amplified. Pitfalls

* *Ruzic-Sabljic 2016. Exp Rev Molecular Diagn*

* **To improve sensitivity: increase the number of targets, up to 8**
  * Early Lyme disease: 62% sensitivity in blood (*Eshoo 2012. PLoS One*)
  * *de Leeuw 2014. J Microbiol Meth.*

* **Classical nested PCR:** higher sensitivity & specificity than standard PCR

* **For clinical samples, larger number of *Borreliae*:** significantly associated with severity of signs & symptoms and positive culture

* **Pitfalls**
  * Inhibitors of PCR:
    * in plasma, CSF, skin, etc.
    * lab products: heparin, formalin
  * Contamination
  * Need for internal control

Lyme Multiplex PCR-dot blot assay

* *Shah et al. (**IgeneX**) 2017. Eur J Clin Microbiol Infect Dis*

* **Whole blood, serum and/or urine**
* **Patients:** suspected Lyme disease
* **Control:** DNA from other bacteria and parasites tested
* **Samples**
  * 107: Houton, Texas
  * 402: IgeneX lab, Palo Alto, California
* **Highly specific** for *B. burgdorferi*
* **Texas:** 21.5% of seronegative samples (CDC's Western blot) are PCR positive
* **IgeneX:**
  * 28.1% of PCR positive samples are CDC's WB positive
  * 58.7% of PCR positive samples are IgeneX WB positive
* **Interest of urine**

Comparison of real-time PCR for *Borrelia burgdorferi s.l.* between five Scandinavian labs

* *Lager 2017. PLoS One*
* **Comparison of 8 PCR methods in 5 labs** (Sweden, Norway, Denmark)
* **Each lab analyses 3 different blinded reference samples**
  * cDNA in water, extracted from cultured *Borrelia*
  * Cultured *Borrelia* in CSF
  * DNA dilutions from cultured *Borrelia burgdorferi* and relapsing fever *Borreliae*
* **Concordance:**
  * in general high,
  * especially between techniques using 16S rRNA as the target gene
  * linked to cDNA as the type of template
* **Sensitivity higher with DNA than cDNA**
* **Some techniques not able to detect *B. spielmanii, B. lusitaniae* or *B. japonica***

Nested real-time PCR

* *Sroka-Oleksiak 2016. Med Dosw Mikrobiol*
* 94 blood samples from suspected Lyme disease

* Gradient of temperature and gradient of magnesium

* Nested PCR real-time: 47.5% positive
* PCR without pre-amplification: 2.1% positive

* Serology: 43.6% positive

Nanotrap* at the early stage of Lyme borreliosis

* *Magni 2015 J Translational Med*
* **Nanotrap* particles concentrate urinary ospA**
* **Particles use a highly specific anti-ospA monoclonal antibody as a detector**
* No homology to human proteins and no cross reactivity with non-*Borrelia* bacterial proteins
* **268 urine samples**
* **Erythema migrans: 24/24 positive** (controls with urine from asymptomatic persons: 0/117 positive)
* **Persistent erythema migrans despite antibiotic treatment: 10/10 positive**
* **Correlation between resolution of signs after treatment and cessation of ospA urinary shedding**
* **July 2018:** Breakthrough Device designation from the **FDA.** Ongoing process before approval for early stage of Lyme disease

Borrelia miyamotoi

Borrelia miyamotoi

* ***Lee SH. 2014. Int J Mol Sci.***
* A highly conserved 357-bp segment of 16S rDNA gene of *B. burgdorferi s.l.* + the correspondent 358 bp-segment of *B. miyamotoi :* amplified by nested PCR (single pair of primers)
* Amplicons used as templates for direct Sanger DNA sequencing
* In winter, spirochetemia in 14 patients, including:
  * Four *B. miyamotoi*
  * One combinaison of *B. miyamotoi* and *B. burgdorferi*

* ***Wroblewski 2017. Ticks Tick Borne Dis.***
* Multiplex real-time PCR. New York state
* 796 clinical specimens (blood & CSF).
  * 8 *B. miyamotoi*
  * 216 *A. phagocytophilum*
  * 10 *E. chaffeensis*

Borrelia miyamotoi : an analysis of 43 cases diagnosed by real-time PCR in France

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Other investigators
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Borrelia miyamotoi

* **In Japan in 2013.** *B. miyamotoi* isolated from patients with Lyme-like disease (*Sato 2014, Takano 2014*)

* **Belgium** (*Cochez 2015*), **England** (*Layzell 2017*)

* **In France:**
  * isolated from 3% of ticks and 5.55% of rodents (*Cosson 2014*)
  * not previously searched in patients

B. miyamotoi in Russia

* ***Karan 2018,*** *Emerg infect Dis*

Isolated from 70 of 473 patients hospitalized for **acute symptoms following a tick-bite**

Methods (1)

* **848 EDTA blood samples**
  * **Control:** 24 healthy sudents
    University of Angers
  * **824 patients** suffering from « SPPT » (« PTLDS »)
    * **« persistent polymorphic syndrome possibly due to a tick bite », close to « post-treatment Lyme disease syndrome »**
    * **living throughout France**

Methods (2)

* **Real-time PCR**
* **Sequence of interest:** 94 bp, located on the glpQ gene
* **Kit specific for *B. miyamotoi* target:** avoids the loss of sensitivity common with multiplex kits
* **Sequencing of some amplification products,** randomly chosen, confirms the specificity of the amplification

Results

* **Healthy controls:**
  * all negative
  * suggests that it is not a commensal

* **824 patients tested: 43 (5.22%) positive for *B. miyamotoi***

* **Of the 43 positive samples:**
  * 21 could be amplified,
  * the 22 others remained below the limit of quantification

Clinical charts of patients with *Borrelia miyamotoi*

* **A standardized questionnaire was retrospectively sent to patients and their physician:** 31 out of 43 responded

* **Duration of symptoms**
  * **Less than one year:** 6 patients
  * **Long term disease:** 24 patients (average 9 years)
    * Among them:

      2 patients > 30 years

      2 patients > 20 years

Lyme serology in 31 patients with *Borrelia miyamotoi*

* **Elisa:**
  * **negative** **in 19 cases** (**76%** of 26 informed cases)
  * doubtful in 3
  * **positive** **in 3**
  * not informed in 6

* **Western blot:**
  * **negative** **in 9 cases** (**50%** of 18 informed cases)
  * **positive** **in 9**
  * not performed (because of negative ELISA) or not informed in 13 cases

Erythema migrans (EM) among *B. miyamotoi* patients

* **Memory of a tick bite: 16/31 (51.6%)**

* **EM previously observed: 5/31 (16.1%)**

Due to *B. miyamotoi* or to a previous *B. burgdorferi sensu lato* infection?

Erythema migrans (EM) among *B. miyamotoi* patients

* **Russian study in early disease post-tick bite:**
  * **3% of patients with EM had a positive PCR for *B. miyamotoi***
    Karan 2018

(compared to **16.1%** of previouly observed EM in our series of chronic *B. miyamotoi* infection)

Clinical presentation of persistent *B. miyamotoi* infection (1)

* **Asthenia:** **31/31 (100%)**
  * Moderate: 10/31 (32.2%)
  * Strong : 21/31 (67.8%) (score of 4 or 5, on a 0 to 5 scale)
* **Joint pain:** **31/31 (100%)**
  * Often migrating
  * Discreet: 9/31 (29%)
  * At high level: 22/31 (71%)
* **Myalgia:** **25/31 (80.6%)**
  * Muscle cramps: 16/31 (51.6%)
* **High level cephalalgia:** **20/31 (64.5%)** (score of 4 or 5, on a 0 to 5 scale)
* **Neuro-cognitive disorders:** **31/31 (100%)**

Clinical presentation of persistent *B. miyamotoi* infection (2)

- **Sleeping disorders:** **31/31 (100%)**
- **Respiratory symptoms:**
  - Chest tightness/lack of air: 13/31 (41.9%)
  - Dyspnea: 6/31 (19.4%)
- **Balance disorders and malaises**
  - Repeated falls: 3/31 (9.7%)
  - Repeated malaises: 2/31 (6.5%)
- **Visual disturbances:**
  - Amputation of the visual field: 1/31 (3.2%)
  - Diplopia: 1/31 (3.2%)
- **Other medical conditions:**
  - Parsonage-Turner syndrome: 2/31 (6.5%)
  - Multiple sclerosis: 1/31 (3.2%)
  - Manic-depressive psychosis: 1/31 (3.2%)

Clinical presentation of persistent *B. miyamotoi* infection (3)

* **Episodes of relapsing fever:** **11/31 (35.5%)**
* **No fever:** **20/31 (64.5%)**
* **Chilliness:** **18/31 (58%)**
* **Hot flushes:** **16/31 (51.6%)**
* **Sweats** (mainly night sweats)**:** **15/31 (48.4%)**

Conclusion. *Borrelia miyamotoi*

- ***B. miyamotoi* found by PCR in 5.22% of French patients suffering from symptoms identified as SPPT « persistent polymorphic syndrome possibly due to a tick bite », close to « PTLDS »:**
  - **43 cases in late stage disease (largest series at this stage)**
  - Majority of negative Lyme serology
  - Asthenia, pain, neuro-cognitive and sleep disorders in 100% of the cases
  - Episodes of relapsing fever in 35.5% of the cases
- **Other large series: the Russian study by Karan 2018:**
  - **Different population of patients; 70 cases in acute phase after tick bite**
- **Need for a large prospective study**
  evaluating PCR in well-defined populations

PCR in SPPT/PTLDS patients. Comparison of different matrices

Pathotique 1 study

* **Patients with SPPT** (persistent polymorphic syndrome after a possible tick bite), close to PTLDS
* Not treated with anti-infective drugs for at least 2 months
* **PCR**
* **Two samples from each matrix, at Day 0 and Day 2**
  * Venous blood
  * Urine
  * Saliva
  * Capillary blood (on a sub-group)

Results of PCR in SPPT/PTLDS patients

* **105 patients**
  * Venous blood, urine, saliva: 71 patients
  * Idem plus capillary blood: 34 patients

* **PCR positive for at least one microbe**
  (Day 0 and Day 2 **or** Day 0 or Day 2):

		Over the limit of quantification
* **Venous blood:**	**12/71 (17%)**	12/12 (100%)
* **Urine:**	**34/71 (48%)**	30/34 (88%)
* **Saliva:**	**64/71 (90%)**	57/64 (89%)
* **Capillary blood:**	**15/34 (45%)**	14/15 (93%)

Ehrlichia

(105 patients, only 34 patients with capillary blood sample)

* **Venous blood (n=5)**
  * plus urine: 1
  * plus urine plus capillary blood: 1
* **Urine (n=6)**
  * plus venous blood: 1
  * plus venous blood plus capillary blood: 1
  * plus saliva: 4
* **Saliva (n=7)**
  * plus venous blood: 1
  * plus urine: 3
  * plus capillary blood: 2
* **Capillary blood (n=3)**
  * plus venous blood: 2
  * plus urine: 2
  * plus saliva: 1
* ***Ehrlichia*: the four matrices seem interesting**

Rickettsia

(105 patients, only 34 patients with capillary blood sample)

* **Venous blood (n=4)**
  * plus urine: 1
  * plus capillary blood: 1
* **Urine (n=16)**
  * plus saliva: 9
* **Saliva (n=21)**
  * plus urine: 9
* **Capillary blood (n=1)**
  * plus venous blood: 1
* ***Rickettsia*: high level of carriage in saliva and infection in urine. No superiority of capillary blood?**

Mycoplasma

(105 patients, only 34 patients with capillary blood sample)

* **Venous blood (n=1)**
  * plus saliva: 1
* **Urine (n=16)**
  * plus saliva: 16
* **Saliva (n=56)**
  * plus urine: 15
* **Capillary blood (n=4)**
  * plus urine plus saliva: 3
* ***Mycoplasma*: high level of carriage in saliva and infection in urine. Superiority of capillary blood**

Candida

(105 patients, only 34 patients with capillary blood sample)

- **Venous blood (n=2)**
  - plus saliva: 1
  - plus capillary blood: 1
- **Urine (n=8)**
  - plus saliva: 4
- **Saliva (n=20)**
  - plus venous blood: 1
  - plus urine: 4
  - plus capillary blood: 2
- **Capillary blood (n=8)**
  - plus urine: 1
  - plus saliva: 2
- ***Candida*. Superiority of capillary blood. Saliva: probable colonization**
- **Urine: possible contamination during urine collection**

Coxiella burnetii

(105 patients, only 34 patients with capillary blood sample)

* **Venous blood (n=3)**
  * plus capillary blood: 1
* **Urine (n=0)**
* **Saliva (n=1)**
* **Capillary blood (n=1)**
  * plus venous blood: 1
* ***Coxiella*: interest of venous blood (capillary blood?)**

Bartonella

(105 patients, only 34 patients with capillary blood sample)

- **Venous blood (n=1)**
  - plus saliva: 1
- **Urine (n=6)**
  - plus saliva: 2
    - one: same species *B. henselae*
    - one: different species *B. henselae* and *B. quintana*
- **Saliva (n=7)**
  - plus venous blood: 1
  - plus urine: 2
    - one: same species *B. henselae*
    - one: different species *B. henselae* and *B. quintana*
- **Capillary blood (n=1)**
- ***Bartonella*: interest of urine. Saliva: possible colonization**

Borrelia

(105 patients, only 34 patients with capillary blood sample)

* **Venous blood (n=0)**
* **Urine (n=2)**
  * only: 2 (*B. hermsii*)
* **Saliva (n=1)**
  * only : 1 (*B. miyamotoi*)
* **Capillary blood (n=0)**
* **Very low sensitivity for *Borrelia***

Babesia

(105 patients, only 34 patients with capillary blood sample)

* **Venous blood (n=5)**
  * plus capillary blood: 1
* **Urine (n=0)**
* **Saliva (n=1)**
* **Capillary blood (n=4)**
  * plus venous blood: 1
* ***Babesia*: interest of venous + capillary blood**

Advantage of a double sample for PCR: at Day 0 and Day 2

	Only one sample positive
* Venous blood:	15.5%
* Urine:	28.8%
* Saliva:	15.6%
* Capillary blood:	30.8%

Single core genome sequencing

Single core genome sequencing. Detection of *Borrelia burgdorferi sensu lato* and Relapsing Fever *Borreliae*

* *Sin Hang Lee, JE Healy, JS Lambert. J Environ Res Public Health 2019*

* **Core genome: all isolates of *Borrelia*.**
  * Highly conserved genus-specific segment of the 16S-rRNA gene
  * *B. burgdorferi s.l.* + Relapsing fever *Borreliae*
  * Comparison with *Borrelia* DNA sequences in the GenBank
  * 21-base PCR primer sites (different sequences from common blood-borne pathogens)

* **Accessory genome: specific of a species**

* **PCR amplicons used as templates for Sanger sequencing** for routine species differentiation

* **Specimens:**
  * venous blood
  * blind-coded serum samples from the CDC (positive or negative)
  * engorged ticks

* **No need of expensive software or bioinformatic expertise** (needed for NGC, next generation sequencing)

Deniers

Do deniers start to change their mind about PCR?

* **IDSA.** *Schutzer SE et al. Clin Infect Dis 2018*
* **French National Reference Centre for Borreliosis & World WHO Reference Centre for Rickettsiosis**

  Eldin, Jaulhac, Mediannikov, Raoult. Med Mal Infect 2019
* « All currently available diagnostic tools are imperfect… »
* « Real-time PCR now plays an important role in the direct diagnosis… »
* « Physicians should always take into consideration the clinical and epidemiological context… »

PCR. Conclusions

* Various techniques, often home-made
* Various results
* Various matrices

* Frequent low sensitivity

* Need for comparative studies and standardization
* Need for a collaboration between human labs and vet labs

* Major problem: in many countries, major labs and reference centres don’t want to work on it