Charité | Campus Mitte | 10098 Berlin

Prof. RNDr. Julius Lukeš
Laboratory of Molecular Biology of Protists
Institute of Parasitology
Czech Academy of Sciences
37005 České Budějovice
Branisovska 31
Czech Republic

Dr. Katrin Kuhls
Institut für Mikrobiologie und Hygiene
Abt. Parasitologie
Dorotheenstr. 96
10117 Berlin

Tel. +49+30+450524028
Fax +49+30+450524902
Katrin.Kuhls@charite.de

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REVIEW

of the PhD thesis "Genetic analysis, evolution and diagnosis of the Old World leishmanias", submitted by Eva Zemanová-Chocholová

Leishmaniases are diseases caused by parasites belonging to the genus *Leishmania* Ross 1903, a member of the order Kinetoplastida, family Trypanosomatidae. This disease occurs in 88 countries on four continents, there have been 12 million reported cases and 350 million people at risk worldwide. There are three major clinical forms of the disease. Cutaneous leishmaniasis (CL) and mucocutaneous leishmanisis (MCL), which together affect 1.5 million people per year, and visceral leishmaniasis (VL), which affects 500,000 people each year. Different species of *Leishmania* parasites, and even different sub-species of parasites, can cause different clinical outcomes and can respond differently to chemotherapy. There is an increasing interest to study leishmaniases, since the epidemiological picture of this disease is changing, mainly because of urbanization, the increasing prevalence of HIV/AIDS, the occurrence of drug resistance, political conflicts such as in Sudan and Afghanistan, and an increase in the number of people travelling from non-endemic to endemic regions. All these factors make leishmaniasis a growing public health concern for many countries around the world.

To date, the number of tools and resources devoted to the diagnosis of *Leishmania* have been limited and in many clinical settings, particularly in low-income settings, diagnosis is based on extrinsic characters and traditional microscopy. Simple, quick and reliable diagnostic tests for species identification are needed particularly for cases in regions where several distinct species are circulating.
The gold standard for species identification, strain differentiation and phylogenetic inference is multilocus enzyme electrophoresis (MLEE). The MON system, based on 15 enzymes, is the most widely used. It is also the basis for the current taxonomy of the genus *Leishmania*. This method has however, several drawbacks: it is time-consuming, requires the cultivation of the parasites, strains with the same phenotype may, in fact, have distinct amino acid sequences, post-translational modifications can influence the electrophoretic mobility, etc. In recent years, DNA-based methods have been used to identify and differentiate the *Leishmania* parasites to overcome these problems.

The present PhD-work uses molecular methods to study Old World *Leishmania* species, especially of the *L. donovani* complex, the causative agent of VL. The phylogenetic relationships between the species of this complex, *L. donovani, L. archibaldi, L. infantum/L.chagasi*, are still not clear, in particular because of conflicting results between isoenzyme and DNA-based approaches. The results of the different approaches applied, are the subject of several publications, which are compiled in this PhD-thesis. In the first publication (Zemanová *et al.*, 2004), random amplified

polymorphic DNA (RAPD) assays have been applied to detect intraspecific diversity for the *L. donovani* complex. 52 primers were applied to 15 strains from different geographical regions, representing 9 zymodemes. The RAPD based trees revealed a significant correlation between genetic polymorphism and the geographic origin of the strains. It has been shown, that Sudanese *L. infantum* MON-30 in fact belongs to *L. donovani*, as was also suggested by other genotyping methods. Mediterranean *L. infantum* strains were strongly monophyletic (with one exception), the Indian and the African strains constituted two monophyletic groups, respectively. The single *L. archibaldi* strain was identified as a member of the African *L. donovani* cluster, and the existence of *L. archibaldi* as separate species has been questioned. The only criticism concerning this RAPD study concerns the small number of studied strains, e.g. only a single *L. archibaldi* strain and a single strain of "*L. infantum*" MON-30 were studied. These single strains are not sufficient for a general phylogenetic conclusion about the validity of *L. archibaldi* and *L. infantum* in East Africa, although other DNA-based studies (following publications compiled in this thesis and published by other authors) have led to the same conclusion.

A good alternative to MLEE is multilocus sequence typing (MLST), because of the numerous drawbacks of MLEE. To this end, the genes coding for the enzymes studied by MLEE have been sequenced. This work has been performed in collaboration by 2 groups: from the Department of Molecular Biology of the University of South Bohemia and the London School of Hygiene and Tropical Medicine. The five metabolic enzymes ICD, ME, MPI, G6PDH, FH have been studied by Zemanová *et al.* (2006), the genes for ASAT, GPI, NH1, NH2, PGD by Mauricio *et al.* (2006). These two works constitute a very important step toward the establishment of MLST as an alternative gold standard for strain typing and species identification for the genus *Leishmania* in general and the *L. donovani* complex in particular. This genotyping method has many advantage over MLEE, e.g. it can be applied directly to clinical samples, it can be used to detect recombination and for population genetics studies. Data generated in different laboratories can be easily compared (which is not the case for MLEE) and deposited in a database. Some indistinguishable MLEE phenotypes were produced by distinct genotypes. Silent genetic polymerphisms provided enhanced discrimination over MLEE, e.g. by subdividing the zymodeme MON-1.
In the work of Eva Zemanová-Chocholová, the above mentioned 5 genes have been sequenced for 27 strains of the *L. donovani* complex. A large number of heterozygous sites has been detected, and though the presence of genetic exchange in *Leishmania* has been supported indirectly. Phylogenetic analysis of the combined sequences (263 kb) showed strong correlation between genotype and geographical origin. The trees based on these enzyme genes confirm in general the tendencies shown by RAPD trees in the previous publication. According to the MLST tree, the main split divides *L. infantum* from Europe and *L. donovani* from Africa and India. Within *L. donovani,* the Ethiopian and Sudanese strains constitute one cluster, the Indian strains a second cluster. The position of a single Indian strain (SC23) remained unresolved. As previously shown by RAPD, *L. archibaldi* was part of the *L. donovani* cluster and its existence as a separate species has been questioned. Also *L. infantum* MON-30 from Sudan was part of the East African *L. donovani* clade. This phenomenon has been clarified recently by Jamjoom *et al.* (2004), who detected that the descriptions of *L. infantum* and *L. archibaldi* from East Africa are a consequence of convergent evolution in the GOT gene, the only diagnostic enzyme locus, that segregates the three species *L. donovani, L. infantum* and *L. archibaldi.* Considering its scale along with the complementary study by Mauricio *et al.* (2006), the work of Zemanová *et al.* (2006) is one of the first attempts to examine intraspecific diversity of protein-coding genes in protozoa. Another important outcome of this work is that it has shown indirectly that recombination might occur in *Leishmania*, because of the abundance of heterozygotes and the presence of mosaic genotypes. This conclusion has important implications for epidemiology and pathogenicity of *Leishmania* parasites.

The work by Lukes *et al.* (submitted) is the summary of the results obtained by different working groups in the framework of the EU-project "Genetic and phylogenetic markers for endemic and resurgent European leishmaniasis". In this work, a multifactorial genetic analysis has been performed based on ~ 18,000 characters for each of the strains studied. Part of these data was obtained by Eva Zemanová-Chocholová using RAPD analysis (presented in Zemanová *et al.* 2004)

and the sequence analysis of the five protein coding genes (Zemanová *et al.* 2006). Other contributions are sequence data of other 5 enzyme genes obtained by Mauricio *et al.* (2006), RFLP data of *gp63* and *cpb* (Quispe-Tintaya *et al.* 2005), sequence data of 3 other protein coding genes and also of some non-coding regions (provided by the group of K. Soteriadou from the Hellenic Pasteur Institute, Athens), and data of 15 microsatellite markers (Kuhls *et al.*, submitted). This combined analysis revealed strong correlation between phylogeny and geographical origin. This study is one of the most extensive attempts to examine the genetic diversity in a group of protozoan parasites. Moreover, the data set that was applied allowed divergence time estimates for *Leishmania* to be performed and to suggest a model of the mode of spread of this parasite. In this joint publication, a new taxonomy for the *L. donovani* complex is proposed, as conclusion of the extensive genetic analyses using different markers performed by several collaborating scientific groups. The results provided by Eva Zemanová-Chocholová are a very important contribution to this work.

Besides the results of genetic analysis providing insight into the evolution, the population structures and the degree of diversity within the *L. donovani* complex, Eva Zemanová-Chocholová focussed on the development of some diagnostic tests for *L. tropica*, *L. major*, *L. infantum* and *L. donovani*. As mentioned above, in some endemic regions there are circulating several species. For a proper treatment it is essential to identify the causative agent at the species level. Especially for field conditions and for laboratories without sophisticated equipment it is important to have quick, easy and affordable tests, directly applicable to biological material. Most of the known diagnostic tests involve RFLP or sequence analysis. Eva Zemanová-Chocholová has developed a new test based on the direct PCR amplification of a species-specific fragment for *L. tropica*, presented in Jirku *et al.* 2006. Several controls have been implemented in the course of test development, e.g. inhibitor, specificity and sensitivity tests. This assay detects less than 5 parasites in the PCR. PCR is negative with DNA of other New and Old World *Leishmania* species, as well as with *Trypanosoma* species. The main advantage of this test is its simplicity. Another potential application of this assay is for epidemiological studies, e.g. identification of infected sandflies and putative host animals.

In another manuscript, which is in preparation, Eva Zemanová-Chocholová presents a multiplex approach for species identification. Primers were designed that selectively amplify species-specific fragments in mixed cultures and infections. The sizes of the amplicons match the requirements of oligochromatography technology. This makes them applicable for this type of quick test in the future. The results of Eva Zemanová-Chocholová are of great importance, because they also enable DNA-based tests in less well-equipped laboratories in countries, where Leishmaniasis is endemic, and because they also strengthen the position of PCR-diagnosis of leishmaniasis in the community of clinicians. The tests have been developed using reference strains and validated using clinical material (also including material from slides and filter paper). Several controls have been implemented, such as negative controls of healthy blood donors, who had never visited regions endemic for leishmaniasis, specificity tests with other species of *Leishmania*, and inhibition controls. Three assays have been developed to distinguish between *L. infantum* and *L. donovani*, two of the assays are based on the different sizes of the PCR product, the third assay is based on species-specific amplification at different annealing temperatures, using species specific forward primers. Another multiplex PCR assay has been developed to differentiate between *L. tropica* and *L. major*. These species can be differentiated by the different size of their PCR products. Concerning this manuscript in preparation the reviewer has the following comments and questions: Since it was shown by other authors that the *L. tropica* populations from different geographical origins as Africa, Middle East and Asia are quite heterogeneous (Schwenkenbecher *et al.* 2005, Schönian *et al.* 2001) it would be useful to include in the specificity tests also strains from different geographical locations, not only from Palestine. The same applies also for *L. major*. Although additional *L. tropica* strains from Turkey and the former Soviet Union have been tested in Jirku *et al.* (2006), it would be useful to include also strains from more distant regions (e.g. Africa). It would be also useful to test more than just one single strain (e.g. of *L. aethiopica*) of other Old World Leishmanias for the specificity tests. In the case of the INF/DON 1 assay it should be considered, that there are *L. donovani* strains from the Middle East, that have microsatellite repeat numbers, that are intermediate between European Mediterranean *L. infantum* and *L. donovani* from East

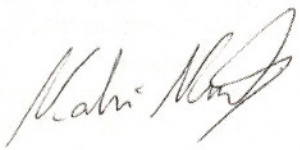
Africa and India and similar to those of a population of *L. infantum* from North Africa. The consequence is that it is very important to include strains from geographically different populations of a distinct species for validation of such a diagnostic test. Table 1 should be improved by adding the WHO-codes for all strains studied, as well as explanations of the abbreviations used. The origin of *L. major* Friedlin has been mixed up. In Table 3 it would be useful to apply the original marker names, if they have been used in previous publications. The figures could be improved by adding the kind of molecular size marker and also the respective fragment sizes.

The whole PhD work is written in English, an additional benefit, since it is accessible for all researchers interested in this field. The author gives a broad overview about the studied parasites, and the methods used for diagnostics and classification. The extensive reference list includes all relevant publications in this field. This general part is followed by the 4 published works, one submitted publication and one publication in preparation, as discussed previously. In the Overview there are some typing errors and some redundancies, which easily can be eliminated. The first chapter about the class Kinetoplastea could be reduced in length, since it contains information, that it is not directly linked with the topic of the PhD thesis. In chapter 2.1 (Clinical symptoms) it would be useful to mention also PKDL as one of the clinical pictures. Since leishmania/HIV co-infections play an increasing role it would be of benefit to add some more information about this issue. In chapter 2.2 (Treatment) the author should add also the drug amphotericine B.
In chapter 3 on classification of the genus *Leishmania* it would be useful to show a scheme of the present taxonomy and to add reference Cupolillo *et al.* (1994) as important contribution for taxonomy of the subgenus Viannia. In chapter 3.1 it should be added that there are different systems for MLEE, e.g. the Montpellier system (MON), the system used for Viannia species in Brazil (IOC), the system of the London School of Tropical Medicine (LON), which all use different sets of enzymes studied. This constitutes an additional complication of this method. *For chapter 3.3 on genotyping it would be of benefit to compile the DNA-based methods (and markers) in a table and to show the taxonomical levels they are useful for, the specificity, sensitivity, if they are applicable for phylogenetic inference and population genetics etc.* The reference part should be checked for correct spelling of the author's names (e.g. Bañuls, Cañavate). It would also improve clearness of the references if the following lines of a paragraph are indented.

The results presented in this PhD thesis of Eva Zemanová-Chocholová constitute important original intellectual contributions to the field of molecular biology of *Leishmania* in general, and on the evolution, population structure, and species and strain differentiation within the *L. donovani* complex in particular. Another important contribution is the development of fast, simple and affordable diagnostic PCR-based tests for *L. major, L. tropica, L. infantum* and *L. donovani.* These tests have the potential to improve the diagnostic routine in countries, in which Leishmaniasis is endemic.

Eva Zemanová-Chocholová has shown in her PhD-work that she is able to design, plan and perform experiments, as well as to interpret and to present the results in an appropriate way. The reviewer appreciates that most of the results have already been published by Eva Zemanová-Chocholová in known international scientific journals.
Her PhD thesis fulfils the requirements for awarding the PhD degree at the reviewer's institution. Eva Zemanová-Chocholová`s thesis would score among the top 25 % among the theses known to the reviewer.
In the opinion of the reviewer, the PhD-thesis presented by Eva Zemanová-Chocholová fulfils the requirements for the PhD title.

The reviewer would like to wish Eva Zemanová-Chocholová all the best for her future scientific work.



Oponentský posudek na disertační práci **Evy Zemanové-Chocholové**
"Genetic analysis, evolution and diagnosis of the Old World leishmanias"

Disertační práce předložená Evou Zemanovou-Chocholovou sestává z dvou částí – literárního úvodu (necelých 20 stran textu a 11 stran citací) a šesti prací v různém stádiu publikování: tří prací publikovaných, jedné práce akceptované, jedné odeslané a jedné připravované k odeslání.

Literární úvod shrnuje současné obecné poznatky o výskytu, přenosu a životním cyklu leishmanií, dále pak uvádí podrobnější poznatky týkající se diagnostiky, evoluce a systematického členění (klasifikace) současných druhů leishmanií. Právě na diagnostiku a odlišení jednotlivých druhů (a komplexů druhů) se zaměřuje druhá část úvodu, včetně charakterizace základních diagnostických metod.
Snaha o genotypizaci starosvětských druhů leishmanií stála u zrodu předkládané disertační práce, která vznikala v širokém konceptu spolupráce řady významných evropských laboratořích zabývajících se výzkumem leishmanií a zapojených do společného projektu EuroLeish. V rámci tohoto projektu se podrobně zpracovávalo nejdříve 15 vytipovaných kmenů a následně 25 kmenů z komplexu *Leishmania donovani*.

Úvod celkem výstižně vysvětluje nejasnosti týkající se problematiky genotypizace i problematiky komplexu druhů starosvětských leishmanií, a vysvětluje tak důvody vedoucí k vypracování disertační práce na toto téma. Objevuje se zde však i několik drobných nepřesností.
Obsah – leishmania x Leishmania
Str. 4 – uvádí se, že leishmanie jsou savčí, současně se ale zmiňuje *L. tarentolae* z gekonů. V kapitole o rodu *Leishmania* by bylo vhodné zmínit již na tomto místě existenci *Viannia*, *Sauroleishmania* atd.
Pojem antropozoonoza není příliš vhodný. (buď antroponoza nebo zoonoza)
Str. (5) a 6 – místo Kala azar je vhodnější používat pojem viscerální leishmanióza (VL) zvláště pokud i ostatní projevy jsou uváděny jako CL, MCL, DCL atd.
U kožní leishmaniozy (CL) není uveden jako původce *L. infantum* a *L. aethiopica*. Navíc zmíněné rozlišení suchých a vlhkých vředů je značně zjednodušené.
U MCL není uveden původce ve Starém Světě.
Str. 8 – sandflies inject the infective stage during blood sucking along with the saliva asociuje, že jsou injikovány ze slinných žlaz, což není pravda. (str. 9 - infective metacyclic forms migrate into the sandfly's proboscis – také není přesné, protože SV je mezi proboscis a MG)
Str. 10 – vzhledem k tomu, že nikde není vysvětleno, co autorka míní rodem *Leishmania*, je nutné do přenašečů počítat i rod *Sergentomyia* (pro *L. tarentolae* aj.)
Str. 14 – u diagnostických metod zcela chybí zmínka o sérologických metodách
Str. 15 – výčet druhů spadajících do podrodů *Leishmania* a *Viannia* zdaleka neobsahuje všechny druhy, dokonce ani ty, které sama autorka jinde v textu zmiňuje.

Citace: není jednotný formát (et al. X *et al.*; Clayton C 1999 x Desjeux, 2004; Maslov *et al.*, 1996 X Maslov and Simpson *et al.*, 1995; Zemanova *et al.*, 2004 and 2006 X Zemanova *et al.*, 2004, 2006; Shanin-Adir et al. 2005 chybí v seznamu literatury – navíc to není nejvhodnější citace k dané problematice atd.)

Druhá část práce sestávající z šesti článků v různém stádiu publikování je uceleným pohledem na problematiku genotypizace a klasifikace leishmanií, a to hned z několika odlišných úhlů. Data obsažená v článcích jsou velmi kvalitní a celý souhrn šesti publikací je skvělou ukázkou jak vytěžit z dané problematiky maximu. Vzhledem k tomu, že celá studovaná problematika byla pro laboratoř molekulární parazitologie pod vedením Dr. Julia Lukeše nová, nezbývá než pochválit autorku a celý tým za množství publikovaných článků i konzistentní přístup ke studované problematice.
Z přiložených článků (v různém stádiu publikace) se zaměřím na ty, kde je předkladatelka prvním nebo druhým autorem. Ačkoliv byly články akceptovány k tisku a prošly náročným oponentním řízením, přesto mám několik poznámek a dotazů.

Zemanová et al. (2004) Genetic polymorphism within the *Leishmania donovani* complex: correlation with geographic origin. *Am. J. Trop. Med. Hyg.* 70: 613-7.

1) Na několika místech v textu (ve výsledcích) se objevuje zmínka o druhu *L. aethiopica*, ačkoliv s tímto druhem dle tab. 1 autoři vůbec nepracovali. Pravděpodobně se jedná o záměnu s druhem *L. tropica*. ???
2) V této, ani v následujících pracích není dostatečně popsána metodika odečtu RAPD vzorů – jak byl odečet prováděn?
3) V tab.1 chybí země původu v popisu kmene MHOM/1993/PM1 (má být MHOM/1993/ES/PM1).
Proč není uvedeno alespoň v poznámce že LON42=MON31.
Vzhledem k diskusi o příslušnosti kmene identifikovaného jako *L. infantum* LG12 (MHOM/SD/1982/GILANI) do skupiny *L. donovani* s.s. by bylo vhodné tuto skutečnost vyznačit do tabulky, jinak se zdá diskuse okolo tohoto kmene částečně bezpředmětná. Proč je tento kmen označován v textu a na obrázcích jako strain MON30? MON30 je zymodem, nikoliv označení kmene (isolátu tedy strain). Všechny ostatní kmeny jsou označovány v textu a na obrázcích kódem nebo názvem kmene, nikoliv příslušností k zymodemu.

Jirků, Zemanová et al. (2006) Development of a direct species-specific PCR assay for differential diagnosis of *Leishmania tropica*. *Diagn. Microbiol. Infect. Dis.* 55: 75-79.

1) Tab. 1 – nejednotné uvádění roku isolace u WHO strain code
2) V kapitole Methods je uvedeno, že citlivost PCR byla testována na kmenu L.t.-SU23, avšak pod příslušným obrázkem (Fig. 1) je uveden jiný kmen (VEDHA).
3) Proč se jako negativní kontrola použila myší tkáň, když experimentální infekce proběhla na křečkovi? (ITS primery specifické na leishmanie například amplifikují rod *Mus*, ale ne rod *Apodemus* nebo *Acomys*)
4) obecný dotaz – v čem hlavně spočívá přínos této nové metody diagnostiky leishmanií v porovnání např. s metodou PCR-RFLP (Schonian et al. 2003)? Domnívám se, že RFLP krok (přidání enzymu do PCR reakce, dokonce bez nutnosti odstranit PCR pufr) není příliš náročný. Navíc v případě že se nejedná o multiplex PCR, má tato metoda v diagnostickém užití značná omezení.

Zemanová et al. (in press) The *Leishmania donovani* complex: Genotypes of five metabolic enzymes (ICD, ME, MPI, G6PDH, and FH), new targets for multilocus sequence typing. *Int. J. Parasitol.*

1) Tab. 1 – Proč je v popisu tabulky vyčleněn *L. infantum* z *L. donovani* komplexu?

Proč není uvedena příslušnost kmenů k jednotlivým druhům (*L. donovani x L. infantum*)? Vzhledem k tomu, že práce je založena na srovnání těchto dvou druhů, považuji neuvedení příslušnosti kmenů k daným druhům za nešťastné. (to samé u tab.1 v článku „Multifactorial genetic analysis of the *Leishmania donovani* complex reveals geographical and evolutionary history, and does not reflect the current taxonomy. (submitted to *Proceedings of the National Academy of Sciences*)".
2) Tab. 2 a Tab 2 pokračování – nejednotnost v kolonce (DIPLO)TYPE
3) Fig. 2 – proč není označena příslušnost k druhům *L. donovani* x *L. infantum* x *L. archibaldi*? Vzhledem k tomu, že se text na toto rozdělení odvolává, je jeho absence ve výsledných obrázcích nevhodná.
4) Autoři vyzívají k ukládání MLST dat do veřejně dostupných databází, jejich vlastní výsledky tam však umístěné nejsou (chybí odkaz na Acc. No.)

Zemanová et al. (in preparation) Towards a multiplex assay for Old World *Leishmania* species identification

Domnívám se, že připravovaný článek je zejména příspěvkem k odlišení leishmanií v komplexu druhů *L. donovani* s.l. a tomuto aspektu by měl být dán větší důraz. Domnívám se, že pro diagnostiku starosvětských leishmanií je metoda PCR-RFLP (Schonian et al. 2003) celkem dostačující a v některých aspektech i vhodnější než multiplexová PCR, avšak metoda PCR-RFLP selhává při jemnějším rozlišení komplexu *L. donovani*.

Obecné dotazy a poznámky:
Přes značné úsilí věnované celé této problematice se domnívám, že počet vybraných kmenů je přece jen nízký a zejména chybí asijské kmeny (jiné než Indické). Tento problém nedostatečného samplingu se může odrazit zejména při vytváření evolučních úvah o vzniku a diverzifikaci starosvětských leishmanií, stejně tak jako úvah o přesunu leishmanióz v důsledku pohybu obyvatelstva.

Mohla by autorka uvést, zda se domnívá, že je systém typizací leishmanií pomocí MONů nahraditelný. A pokud ano, jak konkrétně by měl přechod na nový systém vypadat?

Závěr: Eva Zemanová-Chocholová prokázala, že je schopna kvalitní a samostatné vědecké práce, včetně publikování experimentálních výsledků, i když se domnívám, že by autorka měla klást větší důraz na precizní zpracování svých výsledků. Předložená disertační práce je kvalitní a splňuje podle mého názoru všechna kritéria stanovená BF JČU a plně ji doporučuji k přijetí.

V Praze dne 7. 12. 2006

Jan Votýpka

RNDr. Jan Votýpka, PhD.

Prins Leopold Instituut voor Tropische Geneeskunde
Institut de Médecine Tropicale Prince Léopold
Prince Leopold Institute of Tropical Medecine
Instituto de Medicina Tropical Principe Leopoldo
Stichting van Openbaar Nut | 0410.057.701

Antwerp, 30.11.06

Dr Julius Lukes
Institute of Parasitology
Czech Academy of Sciences
České Budějovice
Czech Republic

Dear Julius,

Please find here enclosed my report on the Ph.D. thesis of Eva Zemanová-Chocholová, entitled "Genetic analysis, evolution and diagnosis of the Old World leishmanias"

I have read with attention the thesis of Mrs Zemanová, whose work I also had the opportunity to follow regularly during the last five years in the frame of the Leishmania genotyping project. I find it a remarkable synthesis covering a broad subject: from phylogenetic analysis of the diversity within the *L.donovani* complex to the development of PCR assays for clinical and epidemiological applications. Mrs Zemanová used independent genetic characters for her analysis (RAPD and sequences of metabolic genes) and obtained congruent results. I also appreciated her revisiting old dogmas of the leishmaniasis taxonomy: i.e. existence of *L.archibaldi* and the attribution of some zymodemes of East Africa to *L.infantum*. Her results clearly support the need for a taxonomical redefinition within this group of parasites. Technologically, significant improvements stemmed from this PhD thesis, like the MLST approach (which could at short term replace the tedious MLEE procedures) and the species-specific PCR assays. In general, I consider the contribution of Mrs Zemanová even more remarkable that it was produced in a laboratory where leishmania is not the first specialty.

Jean-Claude Dujardin • Unit of Molecular Parasitology • Department of Parasitology
Institute of Tropical Medicine • Nationalestraat 155 • 2000 Antwerp • Belgium
Tel. : +32 3 247 63 58 • Fax. : +32 3 247 63 59 • Website : http://www.itg.be

In answer to your 5 specific questions:

1. Does the thesis contain original intellectual contribution?

Yes

2. Is the capability of the student to perform experiments, interpret them and present apparent from the thesis?

Definitively

3. Are the obtained results original? Which are the most important?

Yes, they are. For me, the most original are (i) the taxonomic redefinition within the *L.donovani* complex, (ii) the sequences of the 5 metabolic genes and the MLST approach, (iii) the species-specific PCR and (iv) the global phylogenetic analysis presented in chapter 5

4. Would this thesis be sufficient for awarding the PhD degree at your university/institute?

Yes

5. How would this thesis score among the theses you know off or you have reviewed so far? Would it belong among the top 10%, top 25%, medium or worst 25%?

Top 10%

In conclusion, I declare that the thesis of Mrs Zemanová fulfils the requirements for the PhD title.

I have also a series of questions that might be raised to Mrs Zemanová during her public defence.

A. General introduction:

p.6: being a thesis on taxonomy, a particular care is needed for any taxonomic issue; you mention *L.tropica* and *L.major* as Old World species and later on refer to South American subspecies. I thought that subspecies did not exist anymore. Any clarification on this? What would be your definition of species and sub-species?

p.6: § on VL (comment): Kala-Azar does not produce a dry or ulcering lesion… only parasites belonging to *L.donovani* or *L.infantum* do so

p.8: species identification is not only a matter of taxonomists; it has also a clinical and epidemiological importance. Could you further elaborate on the species-specificity of leishmania response to chemotherapy? Is there a difference between *L.infantum* and *L.donovani* to this respect?

p.12: hybridisation between *L.infantum* and *L.major*. Where do you think hybridisation took place? Vectors of both species are different.

p.17: you mention the operational advantages of species-specific PCR vs PCR-RFLP. However, PCR-RFLP requires a single set of primers and 1 restriction enzyme to type the 5 Old-Wold species, while you would need 5 different species-specific PCR to achieve the same result. Multiplex is an option, but this is extremely difficult to optimalise. Do you believe in a multiplex with 5 pairs of primers?

p.19, last lines. You mention that taxonomies based on different markers are sometimes congruent, sometimes not. Why? Does this not simply depend on the taxonomic level you are looking to?

p.20, general question: your results suggest the need for a taxonomical redefinition of the *L.donovani* complex. However, do you think that genetic information (how good they are) is sufficient for this redefinition? What about epidemiological and clinical information? (If necessary, build-up with next question) Would it not be more important now to (i) go back to the field in East Africa, (ii) undertake a massive study of recent field isolates, well characterised on clinical and epidemiological levels, (iii) type them with your methods, and then (iv) conclude about taxonomy?

B. Paper 1

In my experience, RAPD (specially at intra-species level) needs to be done in triplicate for each samples, and only bands appearing in the 3 aliquots should be considered for the final analysis. This might have influenced the paraphily observed in your report.

C. Paper 2

Did you have an idea of the parasite load in your clinical samples; it is indeed easy to have a high sensitivity with samples rich in parasites…it is an other issue with samples characterised by a low parasite load.

D. Paper 3

- Can you elaborate further on the practical applications of MLST? Do you really believe that this will democratise typing? I personnally think that it will remain a question of few specialised labs. Can you elaborate about alternative methods for genotyping, which might be more democratic? Can you discuss them in the context of different clinical and epidemiological questions? Indeed, genotyping methods must remain adapted to each relevant question.

- Did you use a polymerase lacking 3'-5' proofreading activity on purpose?
- Do you have any explanation for the difference in polymorphism between asat (0.24%) and gpi (0.82?)? Is this significant? Could it have any functional implication?
- In the absence of haplotype frequency, the method chose as ancestral haplotypes those with the most connections. Is this a validated method?
- The type of mosaic structure you mention is inter-gene mosaic. Did you also find intra-gene mosaics? Could a difference in selective pressure on the different genes be an alternative explanation (vs sexual recombination) to the inter-gene mosaic structure?
- the importance of genetic exchange in leishmania remains a controversial issue, and the more discriminatory markers are available, the more controversial it will remain. Where is the limit? Do you think that you have an adequate sample to answer these questions? Why not focusing on sympatric samples to address this issue? Could you compatibilise the current extreme theories on genetic exchange? (comment to further feed the debate) We need to distinguish frequent sexual recombination from rare event followed by clonal propagation of hybrids.

E. Paper 4

- Can you elaborate about non-nuclear genes that might encode similar enzymes as your nuclear targets? Could these non-nuclear genes contribute to the evolution of the nuclear ones?
- Does your figure 2 suggest an 'out of India' hypothesis for the *L.donovani* complex?

F. Paper 5

- You calibrate your evolutionary clock on the *Tbrucei-Tcruzi* split; this is building a hypothesis on another hypothesis. Could it be possible to undertake the calibration, using the *L.infantum-L.chagasi* split, which is more secure with respect to time of divergence (Conquista).
- back to the 'out of India' hypothesis. How do you compatibilise this hypothesis with the observation made by others that current populations of *L.donovani* in the Indian sub-continent are quite homogeneous? Don't you think that your results might be strongly biased by the sample chosen?

G. Paper 6

- Oligochromatography generally permits the detection of only 2-3 different amplicons. Thus it appears difficult to have a real 5-species multiplex in a single stick. From a clinical and

epidemiological point of view, which species should be tackled in priority in a same stick? If needed you can elaborate your answer per geographical region.

Done in Antwerp, the 30.11.2006

Prof. Dr Jean-Claude Dujardin
Head of Molecular Parasitology Unit

INSTITUTE OF TROPICAL MEDICINE ANTWERP
Stichting van Openbaar Nut
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