Leishmania in Sand Flies: Comparison of Quantitative Polymerase Chain Reaction with Other Techniques to Determine the Intensity of Infection

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ABSTRACT Quantification of *Leishmania* parasites in the sand fly digestive tract is important for evaluation of vector competence. We compared quantitative polymerase chain reaction (Q-PCR) with two "traditional" methods, estimation in situ and direct counting with the aid of a hemocytometer, to evaluate their usefulness in different parasite–vector combinations. *Phlebotomus duboscqi* Neveu-Lemarie and *Phlebotomus arabicus* Theodor sand flies were infected with *Leishmania major* and *Leishmania infantum*, respectively, and different approaches were compared to determine the intensity of *Leishmania* infections before and after defecation of the bloodmeal (on days 2 and 8 postinfection, respectively). Estimation of parasite numbers in situ is only a semiquantitative method, but it is quick and provides data about localization of infection. We recommend this technique for low-intensity infections after the bloodmeal is passed. Counting in a hemocytometer is a suitable technique for heavily infected sand flies or for quantification of *Leishmania* within the bloodmeal. Because of its relatively high cut-off (60 parasites per gut), it is not useful for low-intensity infection soon after defecation when parasites are attached to midgut. The most accurate approach for parasite quantification in any type of sand fly infection is Q-PCR. This method is also highly sensitive and can detect one parasite per gut. Localization of a *Leishmania* infection in the sand fly midgut is a parameter equally important to parasite numbers. Therefore, to get full information about the *Leishmania* development in sand flies, we propose to combine various techniques. Both Q-PCR and counting with a hemocytometer always should be preceded by in situ examination under the microscope to assess the localization of the infection.

KEY WORDS sand fly, *Leishmania*, *Phlebotomus*, parasite–vector interaction, RT-PCR

In sand flies experimentally infected with *Leishmania*, and in wild-caught flies from leishmaniasis foci, usually the main parameter recorded is the intensity of parasite infection, i.e., the density of *Leishmania* in the sand fly midgut. The intensity of late-stage infection and localization in the midgut are both thought to be very important parameters for the evaluation of vector competence of a sand fly species or for the developmental success of a particular *Leishmania* strain. However, detailed comparison of data gained by various laboratories is hampered by different methodology, poor methodology, or both used for parasite quantification.

To date, two main methods have been used by various workers to quantify *Leishmania* in the sand fly midgut: direct counting with the aid of a hemocytometer (hereafter hemocytometer counting) and direct estimation in situ under the light microscope. By the first method, midguts of infected sand flies are homogenized in a known volume of phosphate-buffered saline (typically 30–50 μl), and the released promastigotes are counted using a hemocytometer. This method was developed first by Pimenta et al. (1994) for counting promastigotes bound to sand fly midguts in vitro; subsequently, it was used for detection of infection and determination of *Leishmania* numbers in experimentally infected sand flies (Butcher et al. 1996, Kamhawi et al. 2000, Sacks et al. 2000). By the second method, parasite numbers are estimated in situ in dissected midguts under the light microscope, and *Leishmania* density is graded into four to five categories. This approach was used by Killick-Kendrick et al. (1994, 1995) and subsequently, with some modifications, by our group (Cihakova and Volf, 1997, Volf et al. 1998, Sadlova et al. 2003).

Due to recent advances in molecular methods, quantitative polymerase chain reaction (Q-PCR) is routinely used in many laboratories for diagnostic purposes or for the quantification of parasites in host tissue. This technique was described by Nicolas et al. (2002) in *Leishmania*-infected laboratory mice by using kinetoplast DNA as the target for PCR. It proved to be highly sensitive and useful for the diagnosis of canine leishmaniasis (Francino et al. 2006), and for human clinical studies (Mary et al. 2004). As far as we know, Q-PCR had not been used for quantification of *Leishmania* in the sand fly vector before this study.

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The main aim of the current study was to compare these three methods, to investigate their suitability for different parasite–vector combinations, and to discuss their advantages and disadvantages for various applications.

Materials and Methods

Parasites and Vectors. Colonies of *Phlebotomus arabicus* Theodor (origin from Israel) and *Phlebotomus duboscqi* Neveu-Lemarie (origin from Senegal) were maintained at a temperature of 26°C, as described in detail by Benkova and Volf (2007). *Leishmania major* Yakimoff et Schokhor, strain LV561 (MHOM/IL/67/LRC-L137 Jericho II) was maintained on SNB-9 blood agar (Diamond and Herman 1954) supplemented with 50 μg/ml gentamicin. *Leishmania infantum* Nicolle (MHOM/TR/2000/OG-VL) was maintained on RPMI 1640 medium with HEPES (Sigma-Aldrich, St. Louis, MO).

Experimental Infections. Female sand flies (≈7 d old) were fed through a chick-skin membrane on a suspension of promastigotes mixed 1:10 with heat-inactivated rabbit blood. Three sand fly–*Leishmania* models were used. *P. duboscqi* was infected with two different concentrations of *L. major:* 5×10^5 or 5×10^4 cells per ml. In the *P. arabicus–L. infantum* combination, the infective dose was 10^6 cells per ml. Blood-engorged females were separated 1 d postinfection, maintained at a constant temperature of 25–26°C, and sacrificed for dissection on days 2 and 8 postinfection (PI), i.e., before and after defecation, respectively. Midguts were dissected in Tris-NaCl buffer (20 mM Tris and 150 mM NaCl, pH 7.7). In each experiment, 90 dissected midguts were randomly divided into three groups, and parasite numbers in each group of midguts were evaluated by one of the three following methods: estimation of parasite numbers in situ, hemocytometer counting, and Q-PCR.

Estimation of Parasite Numbers In Situ. Individual midguts were placed into a drop of Tris-NaCl buffer, and parasite numbers were estimated under a light microscope at 200× and 400× magnification by an experienced worker. Parasite loads were graded into four categories: negative, 1–100, 100–1,000, and >1,000 parasites per gut.

Hemocytometer Counting. Individual midguts were placed into a microcentrifuge tube containing 30 μl of Tris buffer. Each gut was homogenized with a glass microtissue pestle. Afterward, 30 μl of 2% (vol: vol) formaldehyde in 0.85% NaCl was added, and promastigotes were counted in a hemocytometer under a light microscope at 400× magnification.

Q-PCR. Extraction of total DNA from individual dissected midguts was performed using a DNA tissue isolation kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. DNA was eluted in 200 μl of redistilled water and stored at −20°C. Q-PCR for detection and quantification of *Leishmania* spp. was performed in a Rotor-Gene 2000 from Corbett Research (St. Neots, United Kingdom) by using the SYBR Green detection method (iQ SYBR Green Supermix, Bio-Rad, Hercules, CA). For adequate sensitivity, kinetoplast DNA was chosen as the molecular target, and the primers described by Mary et al. (2004) (forward primer 5′-CTTTTCTGGTCCTCCGGGTAGG-3′ and reverse primer 5′-CCACCCGGCCCTATTTTACACCAA-3′) were used. Two microliters of eluted DNA was used per individual reaction. PCR amplifications were performed in duplicate wells by using the following conditions: 3 min at 95°C followed by 45 repetitive cycles: 10 s at 95°C, 10 s at 56°C, and 10 s at 72°C. The specificity of the reaction was checked in all samples by melting analysis. Quantitative results were expressed by interpolation with a standard curve included in each PCR run. A series of 10-fold dilutions of appropriate *Leishmania*–promastigote DNA, ranging from 10^4 to 0.01 parasites per PCR reaction was used to mix with DNA from females of appropriate sand fly species. DNA from uninfected sand flies was used as a negative control.

Statistics. The Wilcoxon nonparametric test was used to compare differences between results of Q-PCR and hemocytometer counting. To test estimation in situ with the other two methods, we first categorized data from Q-PCR and hemocytometer counting according to the four categories used for the estimation in situ (negative, 1–100, 100–1,000, and >1,000 parasites per gut). The resulting data were analyzed by a chi-square test.

Results

Experimental Infections. We compared two different parasite–vector combinations in three experimental models. *P. duboscqi* females developed heavy infections of *L. major* when infected with 5×10^5 promastigotes per ml of blood. High numbers of parasites were detected before defecation (on day 2 PI) and after this event on day 8 PI (Fig. 1A and B). In the same parasite–vector pair, a 10-fold lower infective dose resulted in low initial infections on day 2 (Figs. 1C and 2). However, after defecation of the bloodmeal (usually on day 4 PI), *L. major* grew well and on day 8 PI colonized the stomodeal valve in ≈60% of infected *P. duboscqi* females (Figs. 1D and 2).

In contrast, the third model, *P. arabicus* infected with 10^6 promastigotes of *L. infantum*, represented a parasite–vector pair with a heavy initial infection on day 2 PI but relatively low parasite numbers on day 8 PI (Figs. 1E and F and 2). The majority of *L. infantum* promastigotes were lost by defecation of the digested bloodmeal; in *P. arabicus* maintained at 25–26°C, this event took place on days 5 or 6 PI. *L. infantum* parasites that persisted beyond defecation grew relatively slowly. On day 8, most infections were still confined to the abdominal and thoracic regions of the midgut, and colonization of the stomodeal valve was detected in only ≈10% of infected *P. arabicus* females.

These three experimental models enabled us to study quantification methods in different situations that may arise during *Leishmania* infection in sand flies.

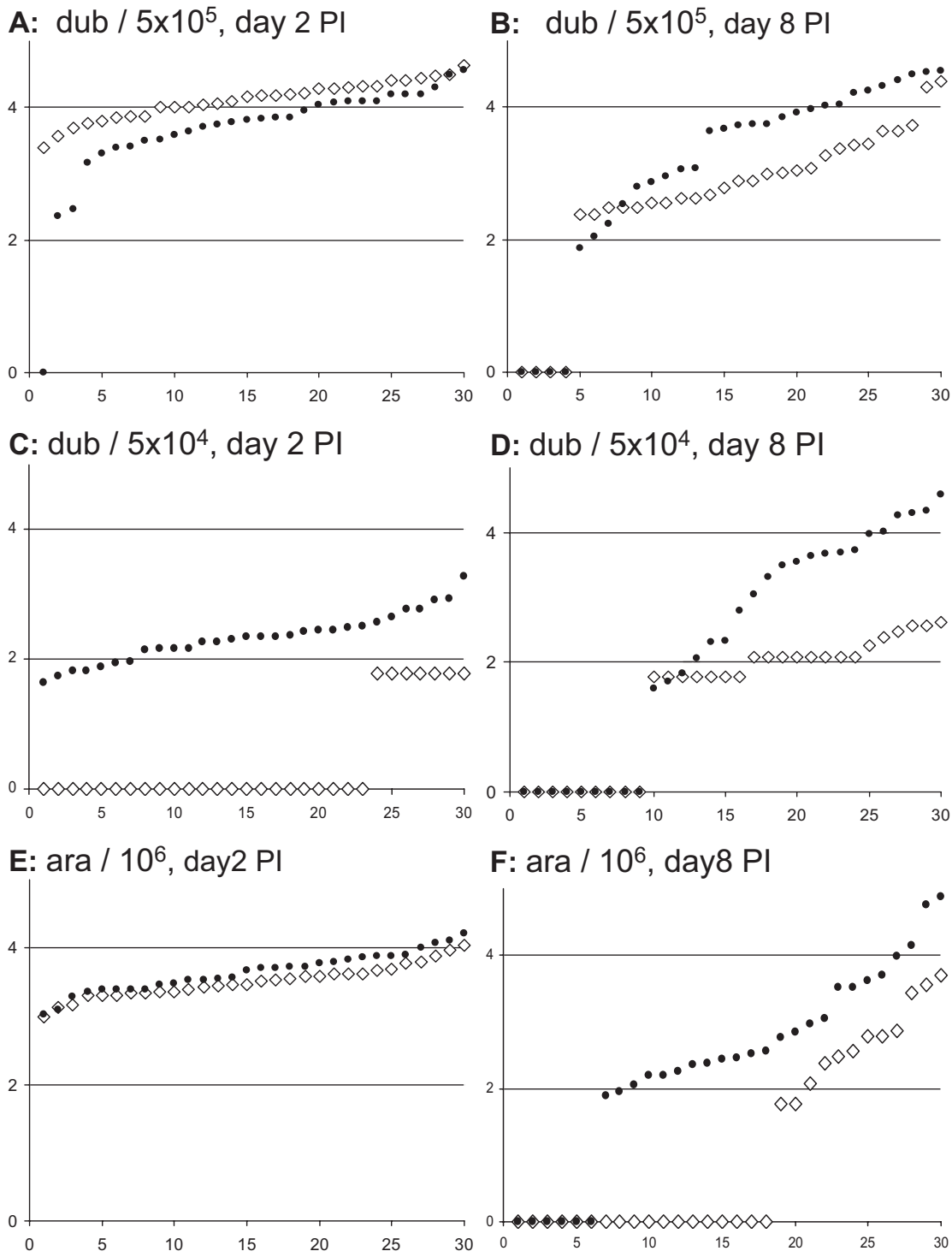

Fig. 1. Comparison of Q-PCR and hemocytometer counting in different parasite–vector combinations. Two groups of 30 midguts were compared in each of six parasite/vector/day combinations. Axes: x, midgut no.; y, $\log_{10}(n + 1)$, where n is number of promastigotes in sand fly midgut counted by two different methods: ◇, hemocytometer, ●, Q-PCR. (A and B) *P. duboscqi*, infected with 5×10^5 promastigotes/ml; day 2 PI, $P = 0.002$; day 8 PI, $P = 0.025$. (C and D) *P. duboscqi*, infected with 5×10^4 promastigotes per ml; day 2 PI, $P < 10^{-10}$; day 8 PI, $P = 0.036$. (E and F) *P. arabicus*, infected with 10^6 promastigotes per ml; day 2 PI, $P = 0.048$; day 8 PI, $P = 0.002$.

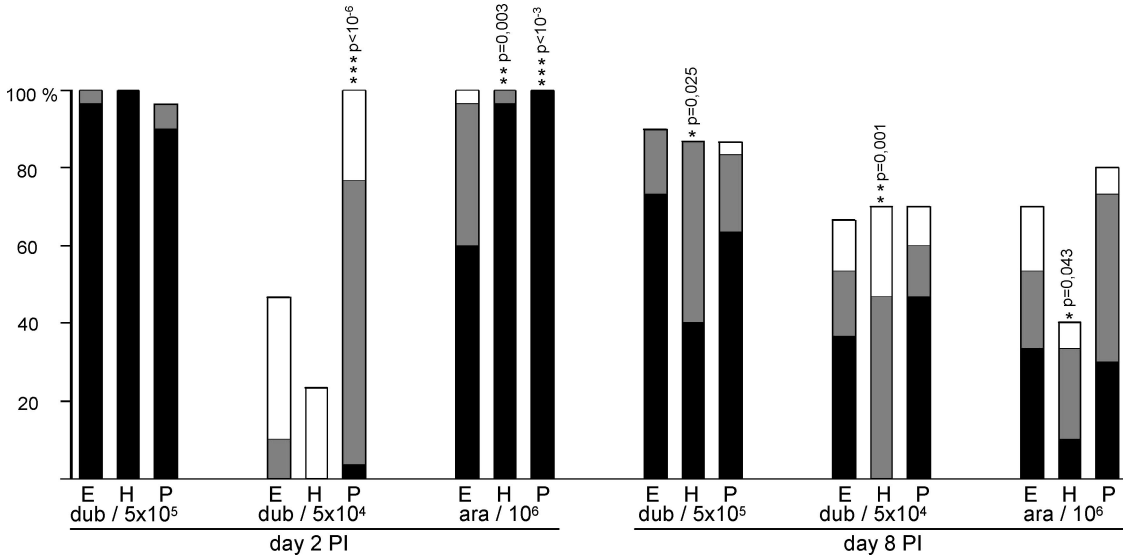

Fig. 2. Comparison between estimation in situ (E), Q-PCR (P), and categorized data from hemocytometer counting (H) in each parasite–vector combinations. Ninety midguts were randomly divided into three groups, and parasite numbers were evaluated by three different methods in each of six parasite–vector–day combinations. Black bar, heavy infections (i.e., >1,000 parasites per midgut); gray bar, medium infections (100–1,000 parasites per midgut); and white bar, light infections (1–100 parasites per midgut). For other abbreviations, see Fig. 1.

Q-PCR. The sensitivity of the Q-PCR method was tested using serial dilutions of parasite DNA extracted from a known number of parasites mixed with midguts dissected from control, noninfected female sand flies. Detection of the kinetoplast DNA of *L. major* was possible down to a level of 0.01 parasites per PCR reaction. This corresponds with one parasite per original sample (two of 200 μl of DNA eluate was used per reaction). This sensitivity allowed a detection limit of one flagellate per dissected sand fly gut and thus confirms this method as suitable for the diagnosis of *Leishmania* in vectors. The dynamic range was up to 10^5 parasites per PCR reaction. To verify the accuracy of the assay for precise quantification of parasites in low numbers, experiments were performed by testing samples with low parasite concentrations (1, 10, 20, 30, 40, and 50 parasites per gut) three times in triplicate (nine replicates). Samples with up to 30 parasites per gut were always PCR-positive, but the numbers of parasites calculated by Q-PCR fluctuated considerably. However, the assignment of parasite numbers at higher concentrations was more reliable. According to our experiments, the detection threshold of Q-PCR is one parasite per gut, and the cut-off for reliable quantification is 30 parasites per gut.

Comparison of Q-PCR with Hemocytometer Counting. The Wilcoxon nonparametric test detected differences ($P < 0.05$) between Q-PCR and hemocytometer counting in all three experimental models and in both intervals studied (Fig. 1). The most significant differences between these two methods occurred in *P. duboscqi* infected by 5×10^4 parasites per ml, where the low infection rate and relatively low parasite numbers occurred before defecation on day 2 PI and after defecation on day 8 PI (Fig. 1C, D).

Comparison of Direct Estimation In Situ with Two Other Methods. On day 2 PI, significant differences between the direct estimation method and Q-PCR were found in two of the three parasite–vector models studied: in *P. arabicus* infected with *L. infantum* and in *P. duboscqi* infected with a low dose of *L. major* (5×10^4 per ml). The difference was more pronounced ($P < 10^{-6}$) in the latter pair (Fig. 2). A difference between estimation in situ and hemocytometer counting was detected only in *P. arabicus* with *L. infantum* (Fig. 2). On day 8 PI, the chi-square test detected a significant difference ($P < 0.05$) between estimation in situ and hemocytometer counting in all three models tested. However, no significant differences were found between estimation in situ and Q-PCR in any parasite–vector pair studied (Fig. 2).

Discussion

To detect different intensities of *Leishmania* infection before and after defecation of the bloodmeal, we exploited various *Leishmania*–sand fly models. *P. duboscqi* is a natural vector of *L. major* as demonstrated experimentally by Lawyer et al. (1990a,b). We infected *P. duboscqi* with two initial concentrations of *L. major*. The concentration of 5×10^5 promastigotes per ml was previously shown to result in high infection rates and high parasite numbers (Cihakova and Volf 1997, Volf et al. 1998). A 10-fold lower infective dose was used to generate low initial infections.

P. arabicus is a vector of *Leishmania tropica* (Wright) in northern Israel (Jacobson et al. 2003); its vectorial competence with this parasite and transmission by bite were demonstrated recently by Svobodova et al. (2006a,b). It is a permissive vector (for

review, see Volf and Myskova 2007), susceptible also to other *Leishmania* species tested, namely, *L. major* and *L. infantum* (Myskova et al. 2007). The *P. arabicus–L. infantum* combination was used to achieve a lower intensity of *Leishmania* infection after defecation. In *P. arabicus* bloodmeal digestion lasts 1–2 d longer than in *P. duboscqi* and 2–3 d longer than in *P. papatasi* (Benkova and Volf 2007). Apparently, in this slowly digesting sand fly, promastigotes of *L. infantum* need a longer time to colonize the stomodeal valve.

The choice of the method for *Leishmania* quantification in the sand fly midgut depends on the purpose of the experiment and on the preferences of the worker. Nevertheless, our experiments enabled us to draw the following conclusions. Estimation in situ is a subjective, semiquantitative technique that requires an experienced worker. However, this method is very quick and enables information to be obtained from a large number of females, which is useful for subsequent statistical analysis. Furthermore, this technique reveals data about the localization of the infection in various parts of the sand fly digestive tract. These parameters, e.g., colonization of the stomodeal valve, are crucial for judging the vectorial status of the sand fly and from the biological point of view, particularly in field studies, are more important than the exact parasite number. However, we can recommend this technique for quantification of *Leishmania* only in those female sand flies that have already defecated their bloodmeals. Before defecation, the semidigested erythrocytes make the estimation difficult and cause serious underestimation of parasite numbers.

The hemocytomer-counting method is supposed to generate accurate information about parasite numbers. However, it is laborious and not suitable for large numbers of sand flies. It quantifies well the parasites within the bloodmeal (before sand fly defecation), but in females that have defecated their bloodmeals, which have low-intensity infections, it gives false-negative results. This is because its detection threshold is 60 parasites per gut (1 μl of the total volume 60 μl is tested in the hemocytometer). Moreover, in contrast to the other techniques studied, it critically depends on precise homogenization of the sample and does not include promastigotes that remain attached to the midgut tissue in the estimate.

The best method for parasite quantification is Q-PCR. Its routine use is complicated by a relatively high cost, mainly due to expensive DNA isolation kits and SYBR Green premix or TaqMan probes. Nevertheless, we suggest the use of this technique in experiments aimed at exact determination of parasite numbers. In such cases, when the number of samples is large it is less laborious than counting by hemocytometer, and, when performed under standard conditions, provides more precise results than the other techniques tested.

In our opinion, quantitative methods should be always accompanied by in situ estimation to determine the localization of the infection under the microscope. The handling of the entire midgut after checking the localization is, however, a delicate operation and part of the midgut with parasites can be easily lost during the transfer from microscope slide into the microtube. This is probably why only one report combining the localization of the infection with hemocytometer counting has been published to date (Nieves and Pimenta 2000). We recommend that researchers in the sand fly–*Leishmania* field combine Q-PCR with determination of localization of the infection and estimation parasite numbers in situ.

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