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Validation of a Microbead-Based Format for Spoligotyping of *Legionella pneumophila*

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A 42-plex clustered regularly interspaced short palindromic repeat (CRISPR)-based typing technique (spoligotyping) was recently developed at the French National Reference Center for *Legionella*. It allows the subtyping of the *Legionella pneumophila* sequence type 1/Paris pulsotype. In this report, we present the transfer of the membrane-based spoligotyping technique to a microbead-based multiplexed format. This microbead-based high-throughput assay uses devices such as Luminex 200 or the recently launched Magpix system (Luminex Corp., Austin, TX). We designated this new technique LP-SPOL (for *L. pneumophila* spoligotyping). We used two sets of samples previously subtyped by the membrane-based spoligotyping method to set up and validate the transfer on the two microbead-based systems. The first set of isolates ($n = 56$) represented the whole diversity of the CRISPR patterns known to date. These isolates were used for transfer setup (determination of spacer cutoffs for both devices). The second set of isolates ($n = 245$) was used to validate the transfer to the two microbead-based systems. The results obtained by the Luminex 200 system were 100% concordant with those obtained by the Magpix system for the 2 sets of isolates. In total, 10 discrepant results were observed when comparing the membrane-based method to the microbead-based method. These discrepancies were further resolved by repeating either the membrane-based or the microbead-based assay. This new assay is expected to play an emerging role for surveillance of *L. pneumophila*, starting with one of the most frequent genotypes, the sequence type 1/Paris pulsotype. However, the generalization of this typing method to all *L. pneumophila* strains is not feasible, since not all *L. pneumophila* strains contain CRISPRs.

Legionella pneumophila is a Gram-negative facultative intracellular pathogenic bacterium, identified as the infectious agent of Legionnaires' disease (LD) in 1977 (1). Several species of the *Legionella* genus are responsible for LD; however, *L. pneumophila* is responsible for the majority of cases of LD, with >90% of all identified clinical cases within serogroup 1, which accounts for ~85% of all cases (2, 3). The organism is quite ubiquitous in aqueous environments, whether natural or artificial (4).

L. pneumophila may replicate in phagocytic protozoa and in human macrophages. Its pathogenicity is related to pulmonary infections, mainly consisting of an acute pneumonia that might be fatal in healthy or immunodeficient patients. As an example, the case fatality rate was 13% in France from 1998 until 2008 (5). Transmission mechanisms are related to environmental factors, such as transmission through contaminated water droplets and airborne transmission.

The European reference method to study the molecular epidemiology of *L. pneumophila* is sequence-based typing (SBT), and clusters are referred to as sequence types (STs) (6). Restriction enzyme analysis using pulsed-field gel electrophoresis (PFGE), although laborious, remains a highly discriminating method and defines clusters referred to as pulsotypes. Other technical choices, such as monoclonal antibody typing and, more recently, multilocus variable-number tandem-repeat analysis (MLVA), have been developed (7). However, such methods have limitations in molecular epidemiological investigations due to their lack of power of discrimination, and they are also tedious and slow to perform. A restriction fragment length polymorphism-insertion sequence analysis method (RFLP-IS) has also been developed and is expected to increase the capacity of molecular investigations of legionellosis outbreaks (8).

In previous work, we showed that in certain cases, some strains that are indistinguishable (either by SBT or by PFGE), particularly within the *L. pneumophila* ST1/Paris pulsotype, could be efficiently classified further using the genetic diversity of their clustered regularly interspaced short palindromic repeats (CRISPRs), and we developed a membrane-based method, spoligotyping (9). The name of this technique is based on that of the original spoligotyping method, which was first used for *Mycobacterium tuberculosis* complex (MTC) (10). Indeed, spoligotyping can be used to design a generic method, since polymorphic CRISPR regions are described at an increased pace and for more and more pathogens (11–13). Spoligotyping gained great acceptance internationally as a first-line method to genotype MTC for molecular epidemiological studies and allowed an understanding of its global phylogeographical structure (14).

Received 29 January 2014 Returned for modification 11 March 2014
Accepted 17 April 2014
Published ahead of print 23 April 2014
Editor: K. C. Carroll
Address correspondence to Christophe Sola, christophe.sola@u-psud.fr.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.00219-14.
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doi:10.1128/JCM.00219-14

The possibility to run spoligotyping on devices other than membranes, such as multiplex microbead-based flow cytometers or fluorescence imager devices, provides a number of advantages. These microbead-based systems allow high-throughput and a better standardization of assays. The multiplexing level is up to 100 for the Luminex 200 system and up to 50 for the Magpix system. These systems are well suited for use in routine analyses and for surveillance and control of infectious disease. Hence, the purpose of this study was to transfer the previously developed membrane-based technique to the microbead-based format, as was done for *M. tuberculosis* and, more recently, for *Salmonella enterica* serovar Typhimurium (12, 15, 16).

MATERIALS AND METHODS

Spoligotyping oligonucleotides and microspheres. The protocol used for the spoligotyping on a membrane has been published previously (9). For the microbead-based spoligotyping, the DR-F primer was labeled at the 5′ end with biotin (instead of digoxigenin [DIG] as for the membrane-based method). All probes were synthesized with a 5′ amino-C_{12} linker (instead of a C_6 linker as for the membrane-based method) to increase the gyration radius (Eurofins MWG Synthesis, Ebersberg, Germany). Microspheres were MagPlex (paramagnetic coated microspheres) when using the MagPix system and MicroPlex (polystyrene microspheres) when using the Luminex 200 system and were purchased from Luminex BV (Oosterhout, The Netherlands). Coupling of the oligonucleotides to the microspheres was performed according to standard procedures, and the PCR was done as previously described (16). Briefly, the total reaction volume was 25 μl per sample: 8 μl of sterilized water, 5 μl of 5× betaine, 2.5 μl of 10× Q buffer [0.2 M Tris-HCl (pH 8.75), 0.1 M KCl, 0.1 M $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 15 mM $MgCl_2$, 1% Triton], both primers (10 μM; 2.5 μl each), deoxynucleoside triphosphate (dNTP) mix (2 mM; 2.5 μl), and 1 U of *Taq* polymerase. Alternatively, another protocol with commercial buffer was also used, in which Q buffer was replaced with 5 μl of 5× Promega Flexi buffer, adding 1.5 μl of 25 mM $MgCl_2$ and 0.2 μl (1 U) of GoTaq from Promega (Mannheim, Germany). The PCR program was as follows: 20 cycles of 95°C for 5 min, 95°C for 20 s, 57°C for 30 s, and 72°C for 40 s. The hybridization took place in a thermocycler at 52°C for 20 min after 10 min of denaturation at 95°C. These steps were performed in a 50-μl volume with 2 μl of PCR product, 15 μl of Tris-EDTA (TE), and 33 μl of bead working solution in 1.5× TMAC (tetramethylammonium chloride) containing around 2,000 beads/analyte on the Luminex 200 or 1,200 beads/analyte on the Magpix. After 5 min of incubation at 52°C in the Luminex analyzer in the presence of 25 μl of 5-ng/μl streptavidin-phycoerythrin (SA-PE; Interchim, Montluçon, France), fluorescence reading was performed by following the Luminex instruction manual and with the use of the Exponent 3 software (Luminex BV, Oosterhout, The Netherlands).

Multiplex analyzers. We used two Luminex high-throughput systems, the flow cytometry-based Luminex 200 system (two lasers) and the fluorescent imager MagPix (Luminex Corp., Austin, TX), with a charge-coupled-device (CCD) camera and light-emitting diodes (LEDs). The oligonucleotide-precoupled MicroPlex and MagPlex beads that were used throughout the study (research use only) are available from our public genotyping Beads4Med services platform within our institute (UMR8621, Institute of Genetics and Microbiology, Orsay, France; http://www.igmors.u-psud.fr).

Clinical and environmental isolates and DNA extraction. The DNA samples studied in this work were provided by the French *Legionella* National Reference Center (NRC) in Lyon. Two sets, totaling 301 DNA samples (set 1 [$n = 56$] for method development and set 2 [$n = 245$] as a validation set), were extracted using the QIAamp DNA extraction kit (Qiagen, Les Ulis, France) from *L. pneumophila* clinical and environmental isolates. The 56 isolates of the 1st set were selected to represent the complete diversity of CRISPR patterns known to date. The 245 isolates of the 2nd set were selected based on the presence of the CRISPR loci. All isolates of the 2 sets have been spoligotyped independently and in a blind fashion by the membrane-based spoligotyping method by the NRC (9). Their membrane spoligotype results were made available once experiments were run in a blind fashion on the Luminex 200 and Magpix systems. The sequence-based typing (SBT) and pulse-field gel electrophoresis (PFGE) genotypes of all isolates had also previously been determined by the NRC. Out of the 301 isolates, 264 isolates had an ST1/Paris pulsotype, 20 isolates had an ST1/non-Paris pulsotype, 11 isolates had a non-ST1/Paris pulsotype, and 6 isolates had a non-ST1/non-Paris pulsotype.

Principle, interpretation of values, statistics, and cutoffs. The principle of interpretation of the LP-SPOL (*L. pneumophila* spoligotyping) raw values obtained on Luminex 200 or on Magpix is the same as the one used previously for other microbead-based methods (17). The positive and negative cutoffs used to interpret whether a spacer is positive or negative were computed using the raw results output file. The isolates of set 1 ($n = 56$) were used in the first step to determine statistically the cutoffs of all spacers. A script previously developed in the R software was applied for statistical calculation (http://www.r-project.org) using a modified receiver operating characteristic (ROC) method. Using these spoligotypes (the set 1, $n = 56$) as reference results, a pair of cutoffs (positive cutoff and negative cutoff) was calculated for each spacer. All cutoffs were determined for the LP-SPOL method experiment on both the Luminex 200 and Magpix systems (Table 1; see also Table S1 in the supplemental material). Once the cutoffs were determined, they were applied to automatically interpret the raw values of each spacer for each new sample by comparison. The positive values of one spacer (presence of that spacer) are values above the positive cutoff, and negative values are those below the negative cutoff. Raw values between the two cutoffs (positive cutoff and negative cutoff) are intermediate and have to be interpreted carefully by an expert, or a sample can be repeated with an increased amount of the PCR product in the analysis. To evaluate the quality of the probes, a signal/noise ratio was calculated by the same script for each probe (Table 1). Signal is considered the mean of raw fluorescence values of isolates in which the spacer is present, and noise is considered the mean of raw fluorescence values of strains, which are devoid of the spacer. Spoligotyping results obtained by the previous membrane-based method for the first 56 isolates were compared to those obtained by the microbead-based method on the same isolates to validate spacer presence or absence and calculation of cutoffs. The second set was run in a blind fashion in light of these cutoffs.

RESULTS

We developed the technique on one set of samples covering the known CRISPR diversity in *L. pneumophila* and validated it using a second independent set. When comparing the quantitative outputs of Luminex (median fluorescence intensities [MFIs]) with the results from membranes of the first 56 isolates, we observed a very strong positive correlation between MFI by the two systems (Luminex and Magpix) and the presence of spacers, except for 13 specific data points for 7 isolates. Indeed, for all spacers but those for 13 of the 56 tested isolates (2,339/2,352 spoligotype data points), high MFI values corresponded to spacers detected as positive (presence of spacer) with the membrane-based method; conversely, low MFI values corresponded to spacers detected as negative (absence of spacer) with the membrane-based method. The 13 discrepant points correspond to low MFI values for 5 spacers positive by the membrane-based method and to high MFI values for 8 spacers negative by the membrane-based method (see Tables S2 and S3 in the supplemental material for MFI values). These results strongly suggested artifacts for the corresponding isolates, and these 7 samples were checked first by rereading the membranes and then, if necessary, by repeating the membrane-based spoligotyping (Fig. 1). These verifications led to the corrections of

TABLE 1 Signal/noise ratio of the 42 probes in the Luminex 200 and Magpix systems[a]

Probe	Luminex 200			Magpix		
	Mean of pos RFU	Mean of neg RFU	Ratio	Mean of pos RFU	Mean of neg RFU	Ratio
Sp1	2,082	47	44	1,313	70	19
Sp2	1,797	76	24	1,489	102	15
Sp3	1,927	97	20	1,723	113	15
Sp4	2,374	244	10	1,841	204	9
Sp5	1,984	42	47	1,511	72	21
Sp6	2,364	57	41	1,830	141	13
Sp7	2,130	63	34	1,689	90	19
Sp8	2,330	76	31	1,815	84	22
Sp9	2,114	80	27	1,730	112	16
Sp10	1,911	73	26	1,537	85	18
Sp11	1,835	153	12	1,434	101	14
Sp12	1,602	116	14	1,398	92	15
Sp13	2,350	60	39	1,748	86	20
Sp14	2,198	97	23	1,647	102	16
Sp15	2,467	81	31	1,916	75	26
Sp16	1,591	111	14	1,263	89	14
Sp17	1,814	58	31	1,475	60	25
Sp18	1,968	67	29	1,617	95	17
Sp19	1,477	63	23	1,108	76	15
Sp20	2,055	62	33	1,689	96	18
Sp21	1,966	58	34	1,601	83	19
Sp22	2,216	64	34	1,910	219	9
Sp23	933	208	4	919	262	4
Sp24	1,861	101	18	1,687	274	6
Sp25	2,547	125	20	2,232	631	4
Sp26	2,233	93	24	1,662	123	13
Sp27	2,082	58	36	1,728	101	17
Sp28	1,623	75	22	1,570	96	16
Sp29	2,097	176	12	1,725	130	13
Sp30	2,033	189	11	1,543	201	8
Sp31	2,336	80	29	1,876	124	15
Sp32	2,101	89	24	1,426	118	12
Sp33	2,523	77	33	1,872	133	14
Sp34	2,522	88	29	1,834	144	13
Sp35	2,532	89	28	2,179	672	3
Sp36	988	86	11	838	100	8
Sp37	2,039	62	33	1,634	116	14
Sp38	2,152	96	23	1,636	111	15
Sp39	1,429	87	16	1,065	95	11
Sp40	2,231	141	16	1,742	301	6
Sp41	1,825	69	26	1,456	88	17
Sp42	1,160	101	12	851	118	7

[a] Fifty-six well-characterized isolates were used. Pos, positive; neg, negative; RFU, relative fluorescence units.

the results obtained by the membrane-based method for all these 13 discrepant points (Fig. 2). The initial discrepancies were due to some errors in the membrane technique, whether due to misinterpretation of hybridization signals (2 isolates), error in transcription of source results into Excel (3 isolates), or a labeling error of the sample (2 isolates) (Fig. 2).

The signal/noise ratios, computed as the ratio between the mean of all positive values and the mean of all negative values for each spacer independently, were high for both systems (see Table S1 in the supplemental material). The negative and positive cutoff values were computed independently for Luminex and Magpix (see Table S1 in the supplemental material). These cutoffs were used to interpret the second set of isolates (validation set). For this set of isolates, 3 discrepant spoligotypes between microbead-based assays and membrane-based assays were also observed. One was resolved by repeating the membrane-based assay; the two others were resolved by repeating the microbead-based assay (Fig. 2). On the validation set, we thus finally obtained 100% concordance for all the unknown isolates ($n = 245$), whatever the method (membrane- or microbead-based assays developed either on the MagPix or on the Luminex 200) (see Table S4 in the supplemental material).

The signal/noise ratio was, on average, higher on the microfluidic laser-based system, Luminex 200, than on the fluorescence imaging magnetic system, Magpix (see Tables S2 and S3 in the supplemental material) (Student paired t test difference = 8.8; df = 41; $P < 0.001$). More data points fell in the gray zone between the maximum of the negatives and the minimum of the positives with the second technique (75, compared to 27 for Luminex 200, out of 12,768 data points). A visual inspection taking into account the quantitative values obtained for the same sample on other spacers could still allow an interpretation of most values. Altogether, the spoligotyping method allowed the identification of 43 different spoligotypes for the 264 isolates that were of the ST1/Paris pulsotype, 5 different spoligotypes for the 20 isolates that were of the ST1/non-Paris pulsotype, 5 for the 11 isolates that were of the non-ST1/Paris pulsotype, and 3 for the 6 isolates

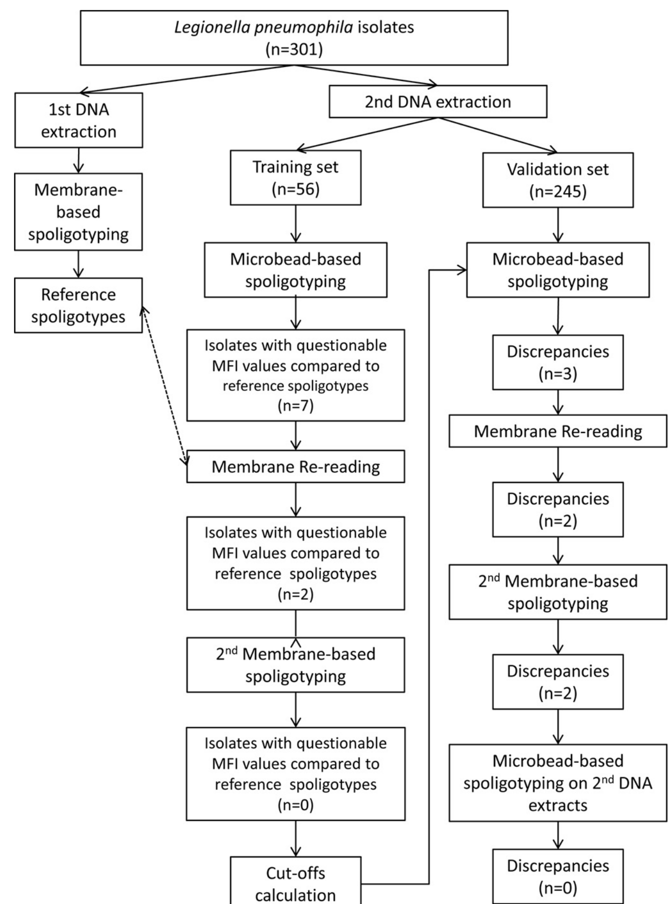

FIG 1 Flowchart of the analytical process.

Study Phase 1 First trial set, blind

n=56 **Identical, n=56; initially different (as shown below), n=7**

Samples	spoligotype microbead-based (Luminex)	spoligotype membrane-based (or sequencing in some cases)
LG 0926 3019		
LG 1127 5020		
LG 1137 1002		
HL 0703 4025		
LG 0713 5007		
LG 0713 5008		
LG 1139 1124		

Samples	Error Analysis after cross talk between the two investigators
LG 0926 3019	spot interpretation error (false positive on the membrane)
LG 1127 5020	spot interpretation error (false positive on the membrane)
LG 1137 1002	identical after check but initial error transcription capture in Excel file
HL 0703 4025	error in tube labeling during the 2nd DNA extraction
LG 0713 5007	error in tube labeling during the 2nd DNA extraction
LG 0713 5008	identical after check but initial error transcription capture in Excel file
LG 1139 1124	identical after check but initial error transcription capture in Excel file

Study Phase 2 Validation set, blind

n=245 **Identical, n=245; initially different (as shown below), n=3**

Samples	spoligotype microbead-based (Luminex)	spoligotype membrane-based (or sequencing in some cases)
LG 0953 4014		
LG 1006 1013		
LG 1036 4010		

Samples	Error Analysis after cross talk between the two investigators
LG 0953 4014	spot interpretation error (false positive on the membrane),
LG 1006 1013	identical after check but initial sample tube inversion error on Luminex (sample 0953-4014 used as sample 1006-1013)
LG 1036 4010	identical after running a second time on Luminex (spacer present)

FIG 2 Description of solution of discrepant data points.

that were of the non-ST1/non-Paris pulsotype. Four spoligotypes were shared by 2 or more of these 4 groups, leading to a total of 51 different spoligotypes for the 301 isolates. STs and pulsotypes of non-ST1 and/or non-Paris pulsotype strains were very diverse; spoligotyping allowed a better differentiation of isolates in these groups only once for 6 isolates with the same ST and pulsotype (ST540/Paris pulsotype), which could be subtyped into 2 spoligotypes (see Table S4 in the supplemental material). None of the 26 non-Paris pulsotype clusters was more discernible by spoligotyping in this study (data not shown).

DISCUSSION

We successfully transferred the membrane-based spoligotyping method of *Legionella pneumophila* to two high-throughput microbead systems (9). The concordance between the microbead-based system and the membrane-based method was virtually complete after careful reexamination of a few discrepant points.

The discrepancies initially observed between the two techniques were due to (i) error in tube labeling during the 2nd DNA extraction, (ii) sample tube inversion, (iii) interpretation errors regarding intensities of spots of the assays obtained by the membrane-based technique, and (iv) error during the transcription of results from the membrane to a digital format. The first two points are technical mistakes that are independent of the methods used. The last two points were linked to the use of the membrane-based method. Similar observations concerning the last two points were made in the study of Abadia et al. (18) that compared membrane-based *M. tuberculosis* spoligotyping results to those from microbead-based systems. Concerning these points, as seen in this study, their study highlights the quality improvement of results by the microbead-based method. This improvement is due first to the better standardization of result interpretation based on quantitative measurements and the use of predefined cutoffs compared to the membrane-based method, for which the interpretation is subjective and operator dependent. It is due also to the absence of manual transcription of results to a digital format, a step during which transcription errors could occur with the membrane-based method.

Until now, and based on whole-genome sequencing of *L. pneumophila* isolates, CRISPR sequences have distinguished 3 classes of *L. pneumophila* isolates: (i) those with no CRISPR, such as Philadelphia, Lorraine, HL 0604 1035, Corby, LPE509, and ATCC 43290; (ii) those with a type IF CRISPR (or linked to IF), as described for the Lens and Alcoy strains (19); and (iii) those with a type II CRISPR, as described in studies on the Paris strains previously sequenced and on the 130b strain (9, 20). The type II

CRISPR was detected in almost 100% of the ST1 and/or Paris pulsotype isolates and in less than 10% of other *L. pneumophila* isolates (9). Among the latter isolates, some harbored different sequences of spacers (124 new spacers could be found in 5 isolates), much more than the 42 used in the currently developed technique (9). Only *L. pneumophila* serogroup 1 isolates of the ST1/Paris pulsotype showed a strong likelihood of CRISPR presence in their genomes. The *L. pneumophila* spoligotyping technique had worked for all isolates of the *L. pneumophila* serogroup 1 sequence type 1/Paris pulsotype (9). Considering the discrimination into 43 different spoligotypes for the 264 undistinguishable ST1/Paris pulsotype isolates, the *L. pneumophila* spoligotyping technique can be recommended as a complementary method for discriminating among these isolates. Spoligotyping allows more discrimination of isolates harboring identical STs and PFGE patterns than does the combination of SBT and PFGE methods. Thus, spoligotyping methods could be developed for the subtyping of other worldwide clinically predominant *L. pneumophila* sequence types harboring one CRISPR locus, such as the ST62, which is now emerging in Germany (21, 22).

The microbead-based system offers the possibility of multiplexing up to 50 analytes in the Magpix, up to 100 in the Luminex 200, and up to 500 in the FlexMap 3D. As the number of *L. pneumophila* genome sequences will increase and the spacer catalog is likely to concomitantly expand, more knowledge obtained through the use of data-mining softwares such as Sipina (http://eric.univ-lyon2.fr/~ricco/sipina.html) and/or Weka (http://www.cs.waikato.ac.nz/ml/weka/) to select the most informative spacers will become feasible and necessary, similar to what has been done for the *M. tuberculosis* model (23). New spacers can be added to the previous set of 42 spacers in a new spoligotyping format that would extend LP-SPOL applicability to all ST1 isolates, all Paris pulsotype isolates, or eventually to other worldwide clinically predominant *L. pneumophila* sequence types harboring one CRISPR locus.

In conclusion, the transfer of *L. pneumophila* spoligotyping techniques to the microbead-based system is an important step that allows (i) a quality improvement in obtained results, (ii) a throughput that is 5× higher (200 isolates in two working weeks with membranes, versus two working days on microbeads), and (iii) a possible implementation to new international public health laboratories and an internationally connected database for surveillance.

The high-throughput and computerized interpretation of results will also promote cost-effective genotyping of a larger number of interesting isolates during epidemiological investigations of *L. pneumophila* outbreaks or of a larger number of environmental samples to prevent new outbreaks. We are currently working on an international evaluation of the microbead-based LP spoligotyping method, beginning with isolates from Italy.

ACKNOWLEDGMENTS

We are grateful to M. F. Topin and J. van Gils from Luminex BV (Oosterhout, The Netherlands) for technical support.

This work was supported by a Ph.D. grant given by Fondation Mérieux and the CNRS to Michel K. Gomgnimbou.

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