Belg. J. Zool., 135 (supplement) : 17-19 December 2005

Pcr detection of *Leptospira* DNA in rodents and insectivores from Tanzania

G.F. Mgode[1], G. Mhamphi[1], A. Katakweba[1], E. Paemelaere[2], N. Willekens[2], H. Leirs[2], R.S. Machang'u[1] and R.A. Hartskeerl[3]

[1] **Pest Management Centre, Sokoine University of Agriculture, PO Box 3110, Morogoro, Tanzania**
[2] **Evolutionary Biology Group, University of Antwerp, Groenenborgerlaan 171, B-2020, Antwerp, Belgium**
[3] **Department of Biomedical Research, Royal Tropical Institute, Meibergdreef 39, 1105 AZ, Amsterdam, the Netherlands**
Corresponding author : G.F. Mgode, e-mail : gfmgode@lycos.com, gfmgode@hotmail.com

ABSTRACT. The true prevalence of leptospirosis in Tanzania is unknown or underestimated. In this study we report on the prevalence of leptospirosis in Morogoro, Tanzania, by PCR detection of leptospiral DNA in 27 kidneys of rodents (*Mastomys* spp, *Rattus* spp, and *Mus* spp) and insectivores (*Crocidura* spp). The PCR study complemented previous attempts to isolate the leptospires and to perform seroprevalence by the microscopic agglutination test (MAT). Results of this study indicated an overall detection rate of 11% by PCR, 7.4% by isolation and 0% by the MAT. Based on our analysis, it is recommended to use PCR and isolation for the detection of leptospires in potential host animals.

KEY WORDS : Leptospirosis, prevalence, microagglutination, rodents, Tanzania

INTRODUCTION

Pathogenic *Leptospira* causes leptospirosis in a wide range of mammalian hosts. Rodents are considered the primary natural reservoirs of leptospirosis in many parts of the world (ALSTON & BROOM, 1958; FAINE, 1982). In Tanzania, the true prevalence of leptospirosis is unknown or underestimated due to limited knowledge on this disease, and hence it is neglected in clinical diagnosis. The gold standard assay in leptospirosis diagnosis is the microscopic agglutination test (MAT) described by WOLF (1954). Due to the diversity of antigens within the *Leptospira* species, this method may fail to reveal infection with certain leptospiral serovars especially in a newly studied area where the prevalent (endemic) serovars are unknown, or in cases where antibody titres are low or absent. Especially in the case of animals, MAT may be specific to an infecting serovar or to antigenically closely related serovars. The chance of detecting leptospiral antibodies in a new environment, therefore, increases with the number of serovars included in the antigen panel.

Leptospires can be isolated from pathological materials (blood, cerebral spinal fluid, urine and kidney tissues), by culturing the primary specimen in selective culture media containing neomycin sulphate, sodium sulphathiozole, cyclohexamide and 5-Fluorouracil to reduce contamination (ADLER et al., 1986; ALEXANDER, 1991; FAINE, 1982). Molecular diagnosis of leptospirosis has been greatly facilitated by PCR detection of specific leptospiral DNA with specific primers that enable amplification of all saprophytic and pathogenic leptospires, as well as leptospires of ambiguous classification (MURGIA et al., 1997; MERIEN et al., 1992; GRAVEKAMP et al., 1993).

The aim of this study was to obtain molecular data for assessing the prevalence of leptospires in Morogoro, Tanzania, by PCR detection of leptospiral DNA from kidney tissues of rodents and insectivores captured in this town. This is the first report on the molecular prevalence of leptospires in an urban/periurban setting in Tanzania.

MATERIAL AND METHODS

DNA extraction from kidney tissues and PCR :

Kidneys of 20 rodents : *Mastomys* spp (18), *Rattus* spp (1) and *Mus* spp (1), and seven insectivores or shrews (*Crocidura* spp) were used. The kidney was ground in a sterile mortar containing 500 µl sterile distilled, de-ionized water. The kidney homogenate (200 µl) was used to extract DNA using the Anansa® Fast 'n' Easy Genomic DNA Purification kit (Tebu-Bio Laboratories, Cedex, France).

The PCR was carried out using specifically designed primers for the detection of pathogenic and saprophytic leptospiral DNA as described by MURGIA et al., (1997) with slight modifications. Briefly, the PCR consisted Lepat 1 (5'-GAG-TCT-GGG-ATA-ACT-TT-3') and Lepat 2 (5'-TCA-CAT-CG(CT)-TGC-TTA-TTT-T-3') primer pair for pathogenic *Leptospira*; and Sapro 1 (5'-AGA-AAT-TTG-TGC-TAA-TAC-CGA-ATG-T-3') and Sapro 2 (5'-GGC-GTC-GCT-GCT-TCA-GGC-TTT-CG-3') primers for saprophytic *Leptospira*.

DNA of known pathogenic *Leptospira* species (serovar Kenya, serogroup Ballum), and saprophytic species (serovar Patoc, serogroup Semaranga) was used as the control.

The reactions mix consisted 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 mM of each deoxynucleoside triphosphates (dNTP), 0.5 µM of each primer, 5 µl template DNA with modified MgCl concentration (2 mM) and DNA polymerase (1 U). The PCR condition for pathogenic *Leptospira* were : initial denaturation at 93°C for 3

min then 35 cycles of denaturation at 93°C for 1 min, primer annealing at 48°C for 1 min, DNA extension at 72°C for 1 min, and further 10 min extension after the last cycle. Saprophytic PCR condition were : heat denaturation at 93°C for 3 min, then 35 cycles of heat denaturation at 93°C for 1 min, primer annealing at 63°C for 1.5 min, DNA extension at 72°C for 2 min and after the last cycle extension continued for further 10 min.

Leptospira isolation from kidney tissues :

Tissue samples were prepared by grinding the freshly obtained kidneys of *Mastomys* spp (18), *Rattus* spp (1), *Mus* spp (1) and *Crocidura* spp (7) in sterile phosphate buffered saline (pH 7.2). About 0.5 ml of the kidney homogenate was inoculated in Fletcher's *Leptospira* medium containing as selective growth inhibitor, 5-Fluorouracil (200 µg/ml). The cultures were incubated at ambient temperature (26-30°C) and examined for leptospiral growth at seven-day intervals by dark field microscopy (FAINE, 1982, 1988).

Seroprevalence of leptospires in rodents and insectivores :

The microscopic agglutination test (MAT) was used to detect leptospiral antibodies in the sera of the same rodents and insectivores used in the PCR and isolation studies. The MAT was carried out as described by COLE et al., (1973) using live antigen of five *Leptospira* serovars. The serovars were; a previously identified/proposed serovar Sokoine (MGODE et al., in Press) (serogroup Icterohaemorrhagiae); serovar Hebdomadis (serogroup Hebdomadis); serovar Hardjo (serogroup Sejroe); serovar Kenya (serogroup Ballum) and serovar Pomona (serogroup Pomona).

RESULTS

PCR of kidney tissues :

Out of 20 rodents and seven insectivores tested, three were PCR positive, generating a 330 base pair product with the Lepat 1 and Lepat 2 primers (Fig. 1). The PCR positive samples were from *Crocidura* spp., 2 of 7 (29%) and *Mastomys* spp., 1 of 18 (6%). None of the samples were PCR positive with the Sapro1 and Sapro2 primers. This finding was consistent with the presence of pathogenic leptospires.

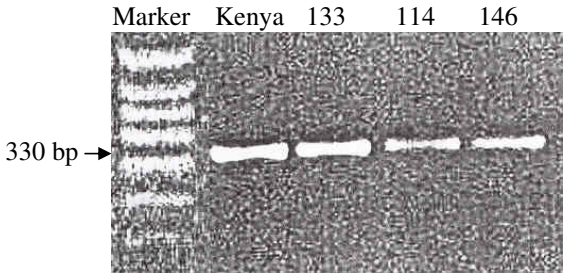

Fig. 1. – PCR products of DNA from kidneys of *Crocidura* spp (133 and 114) and *Mastomys* spp (146) with Lepat1 and Lepat2 primers. Serovar Kenya is the control pathogen, and M is the DNA ladder.
The products were separated by electrophoresis in 3% agarose and stained with ethidium bromide.

Isolation of live leptospires from kidney homogenates :

Kidney cultures of the 27 animals yielded two *Leptospira* isolates from *Crocidura* spp., derived from the same two animals that were scored positively with the PCR method.

Seroprevalence of leptospiral antibodies in rodents :

No agglutination was found in the MAT of the 27 sera against the five-leptospiral serovars used. Table 1 summarises all of these results.

TABLE 1

Comparison of leptospires detection rates by PCR, isolation and microagglutination test (MAT) in rodents and insectivores.

Test	Sample tested	Positive sample	Percent positive
PCR	27	3	11%
Isolation	27	2	7.4%
MAT	27	0	0%

Rodents (n=20) and insectivores (n=7)

DISCUSSION

The results of this study indicated a detection rate of leptospires of 11% (3/27) by PCR on the investigated cases, compared to 7.4% (2/27) by isolation and 0% by serology (MAT). The rate of *Leptospira* detection by PCR per animal species was 6% for *Mastomys* spp. and 29% for *Crocidura* spp. These relatively high infection rates could represent a hazard to public health. As there was only one individual of *Rattus* spp. and *Mus* spp. analysed with the different detection methods, it is not possible to comment on the potential infection rates of these two species. Our data suggest that leptospire detection will be highest using PCR and isolation in a situation where serological antibody detection (MAT) fails, particularly in a new study area.

Serological survey using MAT requires use of known or closely antigenically related prevalent leptospiral serovars. The use of multiple antigens from different serovars in MAT makes this test time consuming. In our study, the negative MAT results found may be due to the limited number of serovars (5) employed as antigen. This also suggests that the leptospires detected by PCR (n=3) and isolation (n=2) might posses a variant antigenic pattern, which is unrelated to that of the new putative serovar Sokoine, serovar Hebdomadis, serovar Hardjo, serovar Pomona and serovar Kenya used in the MAT. However, this is somewhat unexpected as both serovars Machang'u (isolate RM1) and Kenya (isolates Sh9 and Sh25) were recently isolated from cattle and *Cricetomys gambianus* rats in periurban Morogoro, respectively (MACHANG'U et al., 2003). These serovars were thus anticipated to be generally prevalent among rats in urban/periurban Morogoro. An alternative and more likely explanation is that the *Crocidura* spp. and *Mastomys* spp. are natural hosts for one or more of the serovars included in the MAT panel displaying low antibody titres below the detection thresh-

old of the MAT. Lower leptospiral antibody (usually IgG) levels encountered in natural hosts may indeed give MAT negative results, especially in the case of heterologous serovars (BLACKMORE et al., 1984; EVERARD & BENNETT, 1990; PALIT et al., 1991).

The success in isolation of leptospires was supported by the PCR analysis. The percentage of successful isolations may, however, not reflect the true percentage of carriership because a number of cultures (5) were lost due to contamination. Indeed contamination with less fastidious and faster growing microorganisms forms a major limitation of *Leptospira* isolation in spite of the fact that contamination can be kept at minimum by deploying selective growth inhibitors such as 5-Fluorouracil in the culture medium.

As MAT can fail to reveal leptospires carriership in some host animals, it is recommended that PCR and isolation methods are also used, where feasible, to investigate infection sources. These methods are particularly important when carrying out studies in area with unknown leptospirosis prevalence. Rodents and insectivores should be considered important reservoirs of leptospires in the Morogoro area and can serve as indicators of leptospirosis prevalence.

ACKNOWLEDGEMENTS

We thank the Pest Management Centre, Sokoine University of Agriculture (Morogoro, Tanzania), the Evolutionary Biology Group, University of Antwerp (Antwerp, Belgium) and the Department of Biomedical Research, Royal Tropical Institute (Amsterdam, the Netherlands) for supporting this work.

REFERENCES

ADLER, B., S. FAINE, W.L. CHRISTOPHER & R.J. CHAPPEL (1986). Development of an improved selective medium for isolation of leptospires from clinical material. *Veterinary Microbiology*, 12 : 377-381.

ALEXANDER, A.D. (1991). *Leptospira*. In : BALOWS, A., W.J. HAUSLER, K.L. HERMANN, H.D. ISENBERG & H.J. SHADONY (eds), *Manual of clinical microbiology 5th ed.* American society for microbiology, Washington.

ALSTON, J.M. & J.C. BROOM (1958). *Leptospirosis in man and animal.* E & S. Livingstone, Edinberg and London.

BLACKMORE, D.K., L.M. SCHOLLUM & K.M. MORIARTY (1984). The magnitude and duration of titres of leptospiral agglutinins in human sera. *New Zealand medical journal*, 97 : 83-86.

COLE, J.R., C.R. SULZER & A.R. PURSELL (1973). Improved microtechnique for the leptospiral agglutination test. *Applied Microbiology*, 25 : 976-980.

EVERARD, C.O.R. & S. BENNETT (1990). Persistence of leptospiral agglutinins in Trinidadian survey subjects. *European journal of epidemiology,* 6 : 40-44.

FAINE, S. (1982). *Guidelines for the control of leptospirosis*. World Health Organization, Geneva.

FAINE, S. (1988). *Leptospirosis*. In : BALOWS A, W.J. HAUSLER, E.H. LENNETTE, M. OHASHI & A. TURANO (eds), *Laboratory diagnosis of infectious diseases. Principles and practice. Vol. 1. Bacterial, mycotic and parasitic diseases.* Springer-Verlag, New York.

GRAVEKAMP, C., H. VAN DE KEMP, M. FRANZEN, D. CARRINGTON, G.J. SCHOONE, G.J.J.M. VAN EYS, C.O.R. EVERARD, R.A. HARTSKEERL & W.J. TERPSTRA (1993). Detection of seven species of pathogenic leptospires by PCR using two sets of primers. *J. Gen. Microbiol.,* 139 : 1691-1700.

MACHANG'U, R., G. MGODE, J. ASENGA, G. MHAMPHI, R. HARTSKEERL, M. GORIS, C. COX, B. WEETJENS & R. VERHAGEN (2003). Characterisation of *Leptospira* isolates from captive giant pouched rats, *Cricetomys gambianus*. In : SINGLETON, G.R., L.A. HINDS, C.J. KREBS & M.D. SPRATT (eds), *Rats, Mice and People, Rodent Biology and Management.* Australian Centre for International Agricultural Research, Canberra 2003.

MERIEN, F., P. AMOURIAUX, P. PEROLAT, G. BARANTON & I. SAINT GIRONS (1992). Polymerase chain reactions for detection of *Leptospira* species in clinical samples. *Journal of clinical microbiology,* 30 : 2219-2224.

MGODE, G.F., R.S. MACHANG'U, M.G. GORIS, M. ENGELBERT, S. SONDY § R.A. HARTSKEERL (in press). New *Leptospira* serovar Sokoine of serogroup Icterohaemorrhagiae from cattle in Tanzania. *Int. J. Syst. Epidemial Microbiol.*

MURGIA, R., N. RIQUELME, G. BARANTON & M. CINCO (1997). Oligonucleotides specific for pathogenic and saprophytic leptospira occuring in water. *FEMS Microbiol. Lett.*, 148 : 27-34.

PALIT, A., C. HOSKING, L. CREATE, J. MORTON & S. FAINE (1991). Leptospirosis in dairy farmers of Western Victoria, Australia. In : KOBAYASHI Y. (ed.), *Leptospirosis.* Proceedings of the Leptospirosis Research Conference (1990). Hokusen-Sha, Tokyo.

WOLF, J.W. (1954). *The laboratory diagnosis of leptospirosis*. Thomas publishers, USA.