5 DISCUSSION

5.1 Amplification of mtDNA from vertebrate hosts

The PCR was successfully used to amplify homologous segments of mtDNA from vertebrate hosts of tsetse flies, using a universal cytochrome b primer pair (*cytb* 1 and *cytb* 2) directed toward the conserved region. The results reveal that it is possible to employ these primers to amplify a 359 bp fragment of the mitochondrial cytochrome b (*cytb*) gene of the investigated animal species. These results are in agreement with Kocher et al. (1989) and Bellagamba et al. (2001) who reported that universal primers could be used as complements to conserved region of the mitochondrial *cytb* gene in vertebrates.

5.2 Detection limit and stability of host DNA in tsetse

The cytochrome b primers were used to assess the detection limit and stability of vertebrate DNA in the gut contents of tsetse. The results showed that no PCR products were obtained from gut contents of unfed tsetse, which implies that these primers cannot amplify DNA of tsetse origin. This is in line with the reports of Torr et al. (2001) who failed to detect any DNA using these primers in unfed tsetse flies, while they detected cattle DNA in the gut contents of fed flies.

In the present study, it was possible to amplify specific PCR products from blood samples collected up to 96 h post feeding by tsetse flies. This provides some evidence that it is possible to detect DNA from small or incomplete bloodmeals expressed onto filter paper even 72 h after feeding. The present results suggest that the technique is reliable for identifying bloodmeals more than 48 h post feeding, which is in agreement with the report of Boakye and co-workers (1999). Boakye and co-workers (1999) recorded that the host DNA

could be identified up to 3 days following ingestion of the bloodmeal by tsetse. However, the results disagree with Torr et al. (2001) who reported that tsetse in the field completely have digested their meals by 72 h after feeding; during this period the host DNA is degraded and hence reducing the efficacy of the technique.

For the identification and differentiation between different hosts of tsetse, the PCR products obtained from gut contents of flies, fed on sheep blood, were digested with restriction enzyme *Nde*II. We were able to produce restriction fragment profiles up to 96 h after feeding. In addition, the flies were additionally fed on goat blood 48 h after they had fed on sheep blood. When the PCR products obtained from the gut of flies were digested with the same enzyme, only the species-specific restriction patterns of goat DNA were recorded. These results indicate that this technique could be used to detect the last host bloodmeal of tsetse. Similar observations were made by Kirstein and Gray (1996) and Boakye et al. (1999), who reported that PCR-RFLP analysis is a promising method for host species identification of haematophagous insects.

5.3 Amplification of mtDNA after treatment with antiseptic solution

In the present study, it was possible to amplify successfully the mitochondrial DNA of arthropod bloodmeals previously sterilized with aceton, chlorophorm or ether. Treatment of blood samples with antiseptic solutions help to reduce or eliminate the spread of infectious agents through contamination. Boreham (1976) and Linville and Wells (2002) reported that chemical treatment of bloodmeals was able to sterilize the blood without affecting host DNA. Sterilization of bloodmeal prevents spreading of diseases when transferring blood smears from one country to another for testing.

5.4   Amplification of mtDNA from hair and skin of vertebrate species

Amplification of mtDNA from hair or skin samples helps to discriminate the origin of the specimens. In the current study, it was possible to successfully amplify mtDNA samples collected from hair and skin of vertebrate hosts by means of the PCR technique. Hühne et al. (1999) found no differences between the mtDNA sequences of hair and blood samples. Our findings are in line with those of Higuchi et al. (1988) and Schneider et al. (1999). These authors showed that mtDNA from hair could successfully be amplified and give a high degree of individual specificity. For medical anthropologist, the collection of hair samples rather than blood may also be useful in those circumstances where getting blood samples from animals is difficult because of religious or customary beliefs, and where transport of blood samples is difficult or expensive. The melanin pigment, which is present in hair and skin samples, was found to have an inhibitory effect on the PCR. Similar observations were made by Yoshii et al. (1992). To overcome this problem, Bovine Serum Albumin (BSA) was added to the PCR reaction mix, as described by Pfeiffer et al. (1999).

5.5   DNA sequences of different species of the family Bovidae drawn from the
data bank

The sequences of the specific 359 bp fragments of the *cytb* gene of the different animal species of the family Bovidae were drawn from the data bank and aligned for the selection of restriction enzymes. Restriction enzymes used in this study were selected to allow differentiation between the investigated animal species. If the DNA sequence of a respective species is not found in the data bank, the biological significance of the matching result strongly depends on the availability of closely related taxa.

5.6   Identification of the host species of tsetse by PCR-RFLP analysis

The PCR-RFLP technique was used to generate DNA fingerprints for 10 animal species of the family Bovidae by amplifying a 359 bp fragment of the *cytb* gene. The amplified PCR products were digested using three restriction endonucleases, *Taq*I, *Alu*I and *Hind*II, allowing differentiation of the 10 investigated species of the family Bovidae (Figure 17).

Electrophoresis analysis of *Taq*I endonucleas digested amplicons resulted in the expected fragments for the 10 animal species, as revealed in Figure 18. *Taq*I digestion was unsuccessful in discriminating between *Bubalus bubalis*, *Syncerus caffer*, *Hippotragus niger*, *Madoqua kirkii* and *Antidorcas marsupialis* because the sizes of the digested fragments were similar (191-168 and 193-166 bp, respectively). *Taq*I also failed to discriminate between *Bos taurus*, *Kobus leche*, *Ovis aries* and *Oryx gazella* because the PCR amplicons were not digested in all previous species. While *Alu*I endonuclease digestion produced species-specific fragments between the previous species, it was used to distinguish between *Bos taurus* and *Oryx gazella*. This digestion produced characteristic fragments of 190-169 and 304-55 bp, respectively. On the other hand the same enzyme failed to discriminate between *Kobus leche* and *Ovis aries*.

Digestion of PCR products of the 10 animal species with restriction endonucleases *Taq*I, *Alu*I, *Hind*II, *Xba*I and *Hinf*I seemed not to go to completion. In some species, restriction enzymes left some of the original 359 bp DNA fragments undigested; even the use of higher amounts of the enzyme (up to 10 units) and long incubation periods (15 h at 37 °C) could not improve the results. This is in agreement with the work carried out by Céspedes et al. (1998)

and Meyer et al. (1995) who reported incomplete *Alu*I digestion of horse *cytb* DNA amplified by PCR.

Digestion of PCR amplicons of some animal species with a few restriction enzymes resulted in unusual DNA fingerprints in which a greater than expected number of DNA fragments were obtained. Differences between the resultant and the expected fragment sizes for *Ovis aries* (sheep) were also observed. Partis et al. (2000) and Lenstra et al. (2001) reported that variation in these results might be attributed to co-amplification of nuclear *cytb* pseudogenes. Copies of mitochondrial genes may be transferred to the nuclear genome during the course of evolution, and this nuclear pseudogen may also be amplified and analysed by RFLP (Meyer et al., 1995). These pseudogenes are generally not translated and are characterized by an increased number of nucleotide substitutions.

Findings of the unexpected bands during the study are in agreement with those of Partis et al. (2000) who found that the digestion of PCR products with *Hinf*I in sheep (*Ovis aries*) gave unusual DNA fingerprints. These bands had a greater than expected number of DNA fragments obtained following digestion of PCR amplicons with restriction enzyme. The most likely explanation is the co-amplification of nuclear *cytb* pseudogenes (Partis et al., 2000).

5.7   PCR-RFL analysis

Evaluation of the PCR-RFLP approach revealed that it is a simple and reliable technique for tsetse bloodmeal identification. Three different restriction enzymes (*Taq*I, *Alu*I and *Hind*II) were found to be sufficient for the discrimination of all the 10 investigated animal species of the family Bovidae. These findings are similar with those of Kirstein and Gray (1996) who reported that the method could be effectively used for the identification of the zoonotic

reservoirs of lyme borreliosis by analysis of the bloodmeals from the vector (*Ixodes ricinus)*. Work carried by Wolf et al. (1999b) also revealed that the method is reliable for wild and domestic animal species identification.

In comparison to ELISA used in tsetse bloodmeal identification, PCR-RFLP is a much more specific technique. The PCR-RFLP produced species-specific restriction profiles and hence resulted in a clear identification of the different vertebrate animals to the species-level. In contrast, ELISA can lead to cross-reactivity between closely related species (Clausen et al., 1998).

In this study, the sensitivity of PCR-RFLP was determined and later compared with ELISA-sensitivity results of work carried out by Rurangirwa et al. (1986). The results revealed that the sensitivity of ELISA (100-87.5%) was higher than that of PCR-RFLP (80-60%) for samples collected between 40-74 h post-feeding. However, the PCR-RFLP was able to identify tsetse bloodmeals even 96 h after feeding in 40% of the tested samples. This is due to the fact that PCR allows the isolation of sequences from a few copies of intact DNA in extracts where the majority of the molecules are damaged or degraded (Kirstein and Gray, 1996). In addition, the PCR-RFLP was successfully used to amplify DNA from blood samples after treatment with antiseptic solutions (acetone, chloroform or ether).

The method shows promise for the identification of both cooked and uncooked meats. In addition, it can be used to identify the species of autoclaved (120ºC) mixed meat samples to levels of less than 1% (Sun and Lin, 2003). Moreover, hair and skin samples can also be used for the identification of animal species. Partis and co-authors (2000) reported that the method can be used for the identification of almost all vertebrate species.

This approach is potentially more expensive than serological tests (e.g. ELISA) used for bloodmeal identification. The method requires special laboratory facilities for DNA extraction, amplification and avoidance of sample contamination. The materials and reagents used in this technique are more expensive than in the case of ELISA. The estimated cost for the analysis of one bloodmeal sample with PCR-RFLP is approximately € 10 as compared to analysis by use of ELISA, which costs about 35 Cents. The ELISA is routinely used as a field method for identification of bloodmeals of haematophagous insects (Service et al., 1986). Consequently, until the cost of PCR-RFLP reduces considerably, ELISA will continue to be used as a screening test for bloodmeal identification up to the family level. In cases where more detailed information (species level) about the source of bloodmeal is required, the PCR-RFLP analysis can be used for this purpose. The use of these two tests in series will consequently reduce the overall costs that may be incurred when PCR-RFLP analysis is used alone.