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Abstract: Availability of mites is a recognized limiting factor of biological and genetic investigations of the genus Sarcoptes. Current methods of DNA extraction from individual mites also need substantial improvement in efficiency and operator friendliness. We have first developed a technique for efficient and safe extraction of living mites from scabietic skin samples (crusts or deep skin scrapings). Its core device is a large plastic syringe connected with a 1,5 ml Eppendorf tube. The source material is introduced in the syringe and the device in a shoe box with the tip half of the tube emerging. Mites migrate towards a heat source during a minimum of 36 hours. Then, the tube is detached and mites utilized without risks for the operators. A second technique allows operator friendly manipulation of individual mites for DNA extraction. Fixed mites are isolated by adhesion to a small strip of PVC adhesive tape operated with tweezers. Then, mite and strip are plunged in the lyses buffer and the sample twice submitted to thermal shock for disruption of the chitinous exoskeleton. Data show that the tape does not interfere with successive DNA extraction with a commercial kit. The corresponding protocol, that we briefly name "PVC adhesive tape + thermal shock + kit DNA extraction", compares favourably with the available ones.

Two simple techniques facilitate investigation of *Sarcoptes scabiei* individual mites

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Abstract

Availability of mites is a recognized limiting factor of biological and genetic investigations of the genus *Sarcoptes*. Current methods of DNA extraction from individual mites also need substantial improvement in efficiency and operator friendliness. We have first developed a technique for efficient and safe extraction of living mites from scabietic skin samples (crusts or deep skin scrapings). Its core device is a large plastic syringe connected with a 1,5 ml Eppendorf tube. The source material is introduced in the syringe and the device in a shoe box with the tip half of the tube emerging. Mites migrate towards a heat source during a minimum of 36 hours. Then, the tube is detached and mites utilized without risks for the operators. A second technique allows operator friendly manipulation of individual mites for DNA extraction. Fixed mites are isolated by adhesion to a small strip of PVC adhesive tape operated with tweezers. Then, mite and strip are plunged in the lyses buffer and the sample twice submitted to thermal shock for disruption of the chitinous exoskeleton. Data show that the tape does not interfere with successive DNA extraction with a commercial kit. The corresponding protocol, that we briefly name “PVC adhesive tape + thermal shock + kit DNA extraction”, compares favourably with the available ones.

Keywords: individual mite, Sarcoptes scabiei, DNA extraction

A novel extraction technique of living mites from affected skin

Proper and efficient extraction of living *Sarcoptes* spp. mites from the skin lesions of animals affected by clinical scabies is pivotal for: i) the preparation of crude or purified antigen to use in serological diagnostic tests (Bornstein and Zakrisson 1996; van der Heijden et al. 2000; Rambozzi et al. 2004); ii) the isolation of individual mites for genetic studies of the genus, carried out for systematic, epidemiological, physiopathogenetic and therapeutic purposes (Berrilli et al. 2002; Walton et al. 2004; Mounsey et al. 2005; Soglia et al. 2007; Alasaad et al. 2009); iii) the achievement of viable mite aliquots for experimental infections (Bornstein et al. 1995; Lavin et al. 2000; Menzano et al. 2002). Current extraction and concentration techniques are basically inspired to the contributions of Sheahan and Hatch (1975), Andrews (1981) and Nöckler et al. (1992), who described the mass migration of mites off the crusty material following the combined effect of dry heat and vibration, dry heat and light and the sole humid heat, respectively. Common to all methods is the use of disposable Petri dishes as support of the sliced crusts, implying undesired adherence of skin debris, and a final phase in which migrated mites, firmly attached to the dish by their ambulacral suckers, are manually collected by means of fine needles or small blades. This phase is somewhat tedious, time-consuming, often unwelcome to operators due to exposure to a potential zoonotic agent (Beck and Pfister 2006), and may also damage mites intended for experimental infections. Awareness of these drawbacks lead us to develop a complementary solution.

Our source material is represented by skin crusts obtained by free-ranging animals spontaneously dead of hyperkeratotic scabies or euthanized due to this condition. Carcasses are manipulated within 72 hours from presumed death. Basic equipment for mite extraction include: a board box (for instance a shoe box), a 50 cc plastic syringe, a 1,5 cc Eppendorf tube and a reading table lamp (40 Watts light). According to available models, cut of the syringe cone to approx. 5 mm from the base is required. Crusts are divided into pieces about 1 cm^2 wide and 2 g are introduced in the syringe. The plunger is positioned at 15 cc on the scale. The Eppendorf tube is inserted on the syringe cone (Fig.1). A hole corresponding in size to the cylindrical part of the tube is opened on the short side of the box. The syringe/Eppendor tube device (SET) is introduced in the box and kept horizontal (with paper or other packing material) with the conical part of the tube protruding from the hole. The box is kept overnight at 30 cm from the lamp at room temperature. Mites spontaneously migrate in the tubes and may be collected for a minimum of 36 hours, depending on the carcass history. To maximize harvesting, changing the tube is advisable when the cylindrical part is already dotted with mites (sandy appearance, as in Fig. 1). By operating as above described, we have collected material that proved suitable for immunological studies by an indirect ELISA test (Rambozzi et al. 2004), extraction of high quality DNA for genetic studies of individual mites (Berrilli et al. 2002) and successful experimental infection of Alpine chamois (*R. rupicapra*) with the specific *Sarcoptes* strain (Menzano et al., 2002). All drawbacks of the fore mentioned extraction methods seem overcome, since no or minimum debris is passively transported by the migrating mites and no manipulation is necessary beyond detaching and capping the tubes. Finally, a single experiment showed us that the quantity of harvested mites compares favourably with the Andrews's technique (1981). In particular, mite yield per gram of crusty material (the donor was a scabietic Red fox, *Vulpes vulpes*) was 2.1 vs. 0.82 mg (s.d.. ± 0.44 and ± 0.16, respectively; $p < .001$). Compared units (2 g each of crusty material) were six SET devices placed in the same box, and six 150 mm diameter Petri dishes. Extraction time was 24 hours and three more hours were necessary to one experienced operator to collect mites in the Petri dishes. No comparative test for efficiency was done with the methods described by Sheahan and Hatch (1975) and by Nöckler et al. (1992).

A novel protocol for DNA extraction from individual mites

The genetic characterisation of *S. scabiei* requires the development of protocols for efficient DNA extraction from individual mites, that are notoriously small and provided with a hard chitinous exoskeleton. In addition, some Taq-polymerase inhibitors may cause PCR failure (Fischer et al. 2003; Halos at al 2004). Published methods of DNA extraction from pooled mites rely on the use of extraction buffers containing proteinase K and sodium dodecyl sulfate (SDS) in conjunction with phenol extraction and DNA concentration by ethanol precipitation (Zahler et al. 1999). Other protocols combine different mechanical breaking steps of chitinous exoskeleton with protein digestion and use of commercial kits (Weeks at al. 2000; Berrilli et al. 2002; Halos at al 2004; Hill and Gutierrez 2003). We describe here an improved method for extracting sufficient amounts of good quality DNA from individual *S. scabei* mites. Mites were collected from crusty skin lesions of naturally infected Red foxes (*Vulpes vulpes*), Alpine chamois (*Rupicapra rupicapra*) and Alpine ibex (*Capra ibex*) according to both Andrews (1981) and the novel extraction technique of living mites previously described, and then stored in 70% alcohol. Fixed mites were instilled on a glass slide and processed in order to disrupt the exoskeleton using one of the three different protocols subsequently called A, B, and C. T1 (lyses buffer), BE (elution buffer), and proteinase K solution were provided with the NucleoSpin Tissue DNA extraction kit (Macherey Negel, Duren, Germany). In the case of protocol A, used here as a gold standard and processed according to Berrilli et al. (2002), each mite was crushed on a glass slide with the help of a needlepoint and then placed into a 1.5 ml Eppendorf tube. T1 buffer (180 μL) and proteinase K (25 μL) were added and the sample was incubated at 56 °C overnight. Protocol A was applied to 26 mites. In the protocol B, each mite was transferred into a 1.5 ml Eppendorf tube added with T1 buffer (180 μL), with the help of a needle under a dissecting microscope. The sample was then subjected to a heat shock, consisting of a - 80°C step for 2 min followed by a 70°C step for 2 min, repeated three times. The subsequent overnight incubation with T1 buffer and proteinase K was as for protocol A. Protocol B was applied to 26 mites. In the case of the protocol C, each mite was transferred from a glass slide into a 1,5 ml Eppendorf tube added T1 buffer (180 μL), with the help of a small strip of PVC adhesive tape (0.5 x 0.2 mm) operated with a pair of tweezers. Once captured, the mite was plunged in the buffer with the strip. The successive steps were as for protocol B above described, including the heat shock phases. Protocol C was applied to 33 mites. The three protocols shared then the successive steps of DNA extraction using the NucleoSpin Tissue DNA extraction kit (Macherey-Negel, Duren, Germany) according to the manufacturer's instructions. Finally, DNA was eluted with 50 μL of BE buffer. With all protocols, amount and quality of DNA were tested by measuring A260:A280 nm absorbance ratio under a spectrophotometer. The efficiency of the DNA extraction methods was assessed by amplification (PCR) of mitochondrial 16S rRNA gene using 16SD1 and 16SD2 oligonucleotide primers as designed by Walton at al. (2004) in order to amplify a fragment 460 bp long. PCR mixture was prepared in 15 μL final volume containing 0.07 μmol/μL of each primer, 2 mM of each dNTP, 1.5 μL of 10× PCR buffer, 0.75 U of HotSart Taq polymerase (QIAGEN, Hilden, Germany) and 3 μL of DNA salution. PCR was performed in a 2720 GenAmp thermocycler (Applied Biosystem, Courtaboeuf, France) with one 15 min-95 °C denaturation cycle followed by 45 cycles of denaturation (30s, 94°C), annealing (1 min, 50 °C), and extension (1 min, 72 °C); a final 7 min 72 °C extension step was added. Electrophoresis was carried out on 1.5% agarose gels containing ethidium bromide, and DNA fragments were visualized under ultraviolet light. Finally, the PCR fragments were directly cycle sequenced on ABI PRISM 310 Genetic Analyser (Applied Biosystems Foster City, CA) using the BigDay Terminator Cycle Sequening Kit (Applied Biosystems Foster City, CA) and both PCR primers. The resulting sequences were aligned with the Blast program (Altschul et al. 1990) and compared with the 16S gene sequence of *S. scabei* from fox (GenBank Accession Number AF387675). Independently of the protocol, mites provided very similar amounts of DNA (6 ng/μL

on average) of good quality (1.6 A260/A280 ratio; Fig. 2). On the other hand, some differences were found regarding the efficiency of DNA extraction and the compliance of protocols. The shared DNA extraction steps excluded, in protocol A single mite isolation and exoskeleton disruption required on average 30 min, in protocol B the isolation step under a microscope took usually 10-15 min, whereas in protocol C isolation of a single mite took only 5-6 min. A 460 bp PCR product was obtained from 17 out of 26 (65%), 15 out of 26 (58%), and 24 out of 33 mites (73%) processed with protocols A, B, and C, respectively (Chi-squared test on contingency table, $p>.05$). Sequencing and alignment confirmed that the fragments corresponded to the expected 16S rRNA gene region. Based on these results, the PVC strips did not seem to interfere with DNA extraction or with PCR. Although not statistically significant, some difference in favor of protocol C may be recognized, probably due to the imperfect unintentional plunging of some needle-transferred mite into T1 buffer in protocol B, a drawback unlikely to occur with mites once adhered to the PVC strips. Finally, the time needed for isolation of individual mites was slightly reduced in protocol C, then operators reported feeling much more comfortable with the use of PVC strips instead of the needle.

In conclusion, protocol C (PVC adhesive tape + thermal shock + kit DNA extraction) that we have established, improves the efficiency and user-friendliness of the corresponding DNA extraction protocols published so far.

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Figure Legend

Fig.1 Scheme of the operating syringe/Eppendor tube device (SET in the text). Three scabietic crusts (in grey) are visible in the syringe. Several mites (small circles) have already entered the tube.

Fig.2 16S PCR result of DNA extracted with protocol A (line 2 to 4) and protocol B (line 6 to 8): in to break the exoskeleton in the protocol A the individual mite was pierced directly on the slide with the help of the needlepoint, in the protocol B the mite was subjected to heat shock.

fig1
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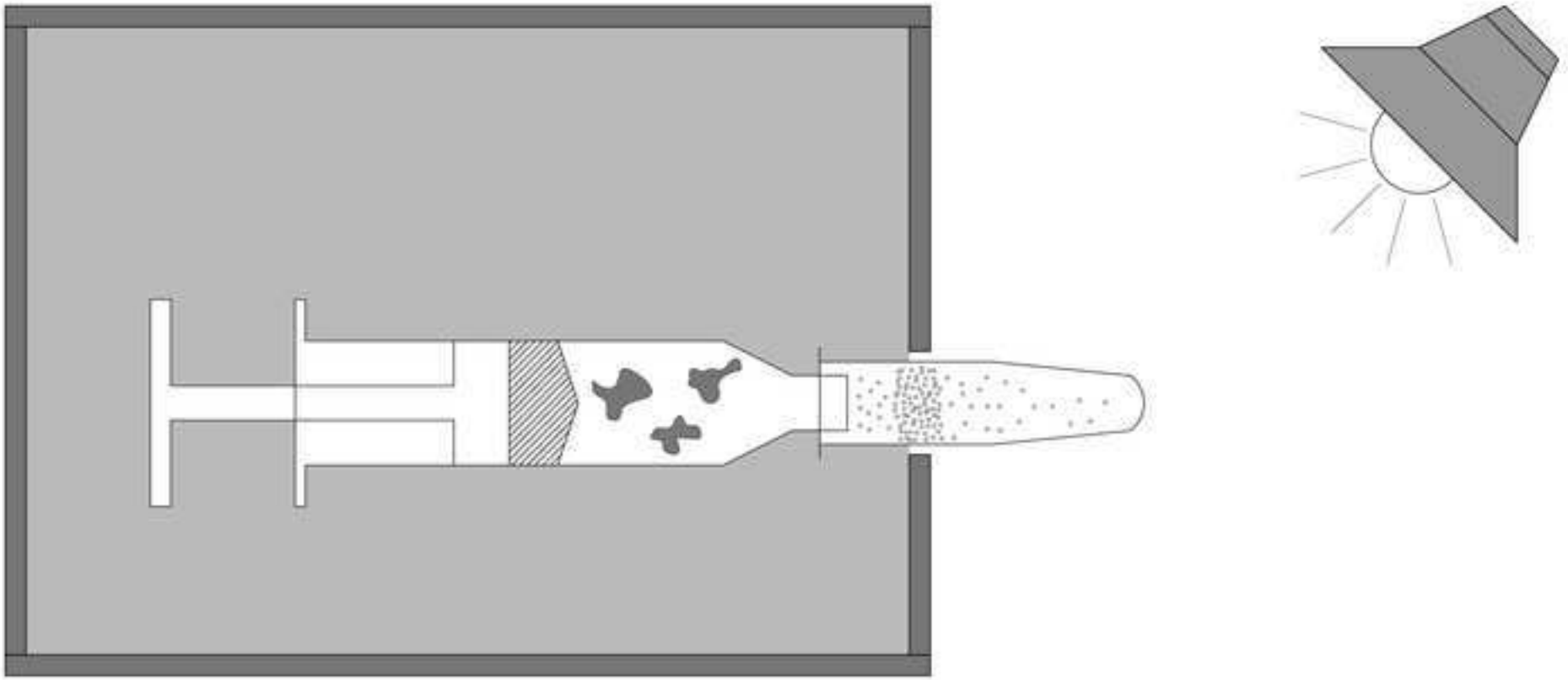

fig2

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