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Abstract

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Short Communication

Effectiveness of Postponed Isolation (Post-frozen Isolation) method for PCR-quality *Sarcoptes* mite gDNA

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Abstract

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Keywords: *Sarcoptes scabiei*, Sample collection method, Direct Isolation (Live Isolation), Postponed Isolation (Post-frozen Isolation), Genomic DNA, DNA extraction

Introduction

Genetic research on *Sarcoptes scabiei*, a worldwide distributed mite with a broad host range including more than 104 mammalian species, most of them free-ranging (Bornstein et al. 2001), has been extremely limited compared with other parasitic agents; this is primarily due to difficulties in obtaining suitable skin samples from different hosts, isolating sufficient numbers of mites and extracting adequate amounts of genetic material from individual mites (Walton et al. 2004). Due to inefficiencies adding at several points, variable proportions of failed preparations have been reported, up to above 50% for some batches (Walton et al. 1997; Berrilli et al. 2002). Focussing on *Sarcoptes* varieties spreading in free-ranging wildlife, additional drawbacks derive from the life-history of samples, often opportunistic ones collected in remote areas (Dagleish et al. 2007) and then submitted to unpredictable transport stresses before mites collection (Walton et al 1997). Studies on these varieties would clearly benefit from use of freezed skin as source of individual mites. However, this possibility has not been explored so far to the best of our knowledge. Mite DNA extraction was usually reported from fresh skins, using somewhat labour-intensive and time-consuming techniques (e. g. Brimer et al. 1993; Smets and Vercruysse 2000; Shanks et al. 2000; Skerratt et al. 2002), with their associated risk of field personnel infestation (Skerratt and Beveridge 1999; Menzano et al. 2004). Only Zahler et al. (1999) reported on mass DNA extraction from skin scrapings, not individual mites, previously frozen in liquid nitrogen and then ground to a fine powder in a mortar, but quantitative details on the efficiency of the technique were not provided. Hence, aim of this technical note was to estimate if individual *Sarcoptes* mites extracted from freezed crusty skin (Post-frozen Isolation method) are suitable

sources of PCR-quality genomic DNA, and to test the effectiveness of this method comparing with, the commonly used, Direct Isolation method.

Materials and methods

Collection of *Sarcoptes scabiei*

Adult mites were individually extracted from the skin of different European free-ranging animals (Alpine chamois *Rupicapra rupicapra*, Red fox *Vulpes vulpes*, Alpine ibex *Capra ibex* and European moufflon *Ovis aries musimon* from Italy; Southern chamois *Rupicapra pyrenaica*, Iberian ibex *Capra pyrenaica* and again Red fox from Spain). Of a total of 510 *Sarcoptes* mites: (1) 250 mites were obtained by placing pieces of freshly collected crusted skin or skin scrapings in Petri dishes under a 40W electric light bulb overnight (Sheahan and Hatch 1975). The living mites having migrated to the underside of the Petri dish were fixed in 70% ethanol and stored at room temperature before the DNA extraction. This protocol was labelled as LI (Live Isolation); (2) 260 *Sarcoptes* were obtained from –20°C freezed crusted skin or skin scrapings. Freezed crusts were defrosted and diluted in tap water, and the dead mites collected with a needle under a dissecting microscope. Fixation and conservation were similar as in 1). This protocol was labelled as PFI (Post-frozen Isolation). Mites were identified as *S. scabiei* according to the morphological criteria of Fain (1968).

Preparation of *Sarcoptes* gDNA

NucleoSpin Tissue kit procedure (Macherey-Nagel, Düren, Germany) was applied for preparation of mite gDNA from all single *Sarcoptes* samples in this study (collected with Direct Isolation or Postponed Isolation methods), according to the manufacturer's recommendations. The resulting volume of each single mite sample was 50 μL.

Extracted DNA validation

Fluorescent-based PCR analysis of microsatellite DNA

From the panel described by Walton et al. (1997) 10 microsatellites (Sarms 33, 34, 35, 36, 37, 38, 40, 41, 44 and 45) were selected and applied with one multiplex 10x PCR as reported by Soglia et al. (2007). Each 15 μL PCR reaction mixture consisted of 3 μL of the single *Sarcoptes* DNA (resulting of Direct or Postponed Isolation), together with the PCR mixture containing all primer pairs (ranging from 0.04 μM to 0.1 μM per primer), 200 μM of each dATP, dCTP, dGTP and dTTP, 1.5 μL of 10X PCR buffer (200 mM KCl and 100 mM Tris-HCl, pH 8.0), 1.5 mM $MgCl_2$ and 0.15 μL (0.5 U/reaction) HotStartar *Taq* (QIAGEN, Milano, Italy). Samples were subjected to the following thermal profile for amplification in a 2720 thermal cycler (Applied Biosystems, Foster City, California): 15 min at 95°C (initial denaturing), followed by 37 cycles of three steps of 30 s at 94°C (denaturation), 45 s at 55°C (annealing) and 1.5 min at 72°C (extention), with a final elongation of 7 min at 72°C.

Microsatellite analysis

Using 96-well plates, aliquots of 12 µL of formamide with Size Standard 500 Liz (Applied Biosystems, Foster City, California) and 2 µL PCR product, were prepared; then the plates were heated for 2 min at 95°C and chilled to 4°C. Fluorescent PCR amplification products were analysed by ABI PRISM 310 Genetic Analyzer with POP 4 (Applied Biosystems, Foster City, California).

Second internal transcribed spacer (ITS-2) of rRNA gene

The Second internal transcribed spacer ITS-2 (~450 bp) was amplified by PCR using primers RIB-18 and RIB-3 as reported by Zahler et al. (1999). PCR was carried out in a final volume of 15 µL. The standard PCR reaction contained 3 µL of the single mite DNA (Direct or Postponed Isolation), 200 µM of each dNTP, 1.5 µL of 10X PCR buffer (200 mM KCl and 100 mM Tris-HCl, pH 8.0), 0.5 mM $MgCl_2$ and 0.15 µL (0.5 U/reaction) HotStartar *Taq* (QIAGEN, Milano, Italy). Samples were subjected to the following thermal profile for amplification in a 2720 thermal cycler (Applied Biosystems, Foster City, California): 15 min (initial denaturing) at 95°C, followed by 45 cycles of three steps of 30 s at 94°C (denaturation), 1 min at 60°C (annealing) and 1 min at 72°C (extention), with a final elongation of 7 min at 72°C. The electrophoresis was performed on 1.5% agarose gel stained with ethidium bromide for DNA visualization under UV light.

Yield of soluble DNA

The yield of soluble DNA was measured, only for successful reactions, by A260:A280 nm absorbance ratio under a spectrophometer.

Statistical procedure

Fisher's exact test and ANOVA test were performed with SPSS software (SPSS 15.0S for Windows, 2003) and the null hypothesis was rejected at $\alpha < 0.05$.

Results

Mites obtained by DI or PFI were tested as sources of PCR-quality gDNA by amplifying a ~450 bp product (ITS-2) and by multi-locus 10x genotyping using microsatellite markers. Overall, 5610 locus-specific reactions were carried out (510 ITS-2 and 5100 microsatellite reactions). With Direct Isolation method, 523 (48 ITS-2 and 475 microsatellite) reactions out of 2750 (19%) failed to be amplified, while for Postponed Isolation, 973 (88 ITS-2 885 and microsatellite) preparations out of 2860 (34%) were unsuccessful. The difference was significant ($P < 0.001$) (Fig. 1). The average yield of soluble DNA was 5.08±1.36 µg/µL (ranging from 4 to 9 µg/µL) following DI, and 4.92±0.97 µg/µL (ranging from 4 to 7 µg/µL) by PFI, with no difference between methods ($P = 0.189$) (Fig. 2).

Discussion

Data show that *Sarcoptes* Post-frozen Isolation method is as good as Direct Isolation method regarding the yield of soluble DNA. Likewise, a large proportion of dead mites collected from freezed skin samples is suitable for *Sarcoptes* gDNA preparation (66 %). This ratio of successful reactions, 66%, is to be considered high, comparing with those reported in mite literature (Walton et al. 1997; Berrilli et al. 2002). The Direct Isolation

method has 15 % higher chance of success comparing with Post-frozen method. Notwithstanding, the availability of skin samples suitable for DI is definitely less frequent due to obvious environmental and logistic difficulties, amongst them is the understandable reluctance of field personnel, made aware of the zoonotic risk associated with exposure to mites of animal origin, resulting from the manipulation of fresh scabietic carcasses (Bornstein et al. 2001; Menzano et al. 2004), which obviously compensate the difference in the ratio of successful reactions in favour of PFI. Under the above circumstances (a wide majority in our personal experience) freezing the whole carcass or a large skin sample following minimum glove-mediated contact is more welcome and accepted protocol. The Post-frozen Isolation method is an effective and recommended method for mite gDNA preparation, especially when we expect to come into equilibrium among profitability, practicability and safety in systematic *Sarcoptes scabiei*.

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Figure legends

Fig. 1 Representative samples showing the effect of *Sarcoptes* mite collection method (Direct Isolation and Postponed Isolation) on the amplification of Sarms-41 fluorescent-based microsatellite DNA. 'Control' represents no-DNA control.

Fig. 2 Effect of *Sarcoptes* mite collection method (Direct Isolation and Postponed Isolation) on the yield of soluble DNA.

Fig. 1

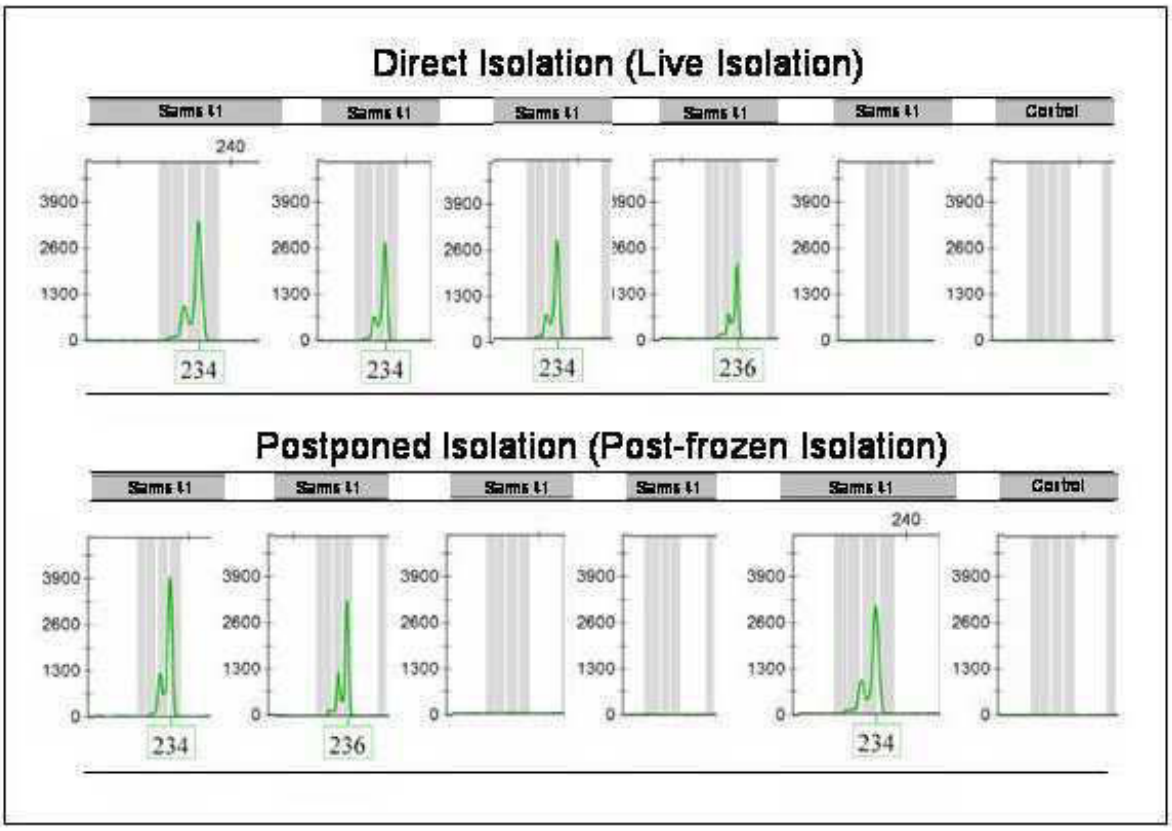
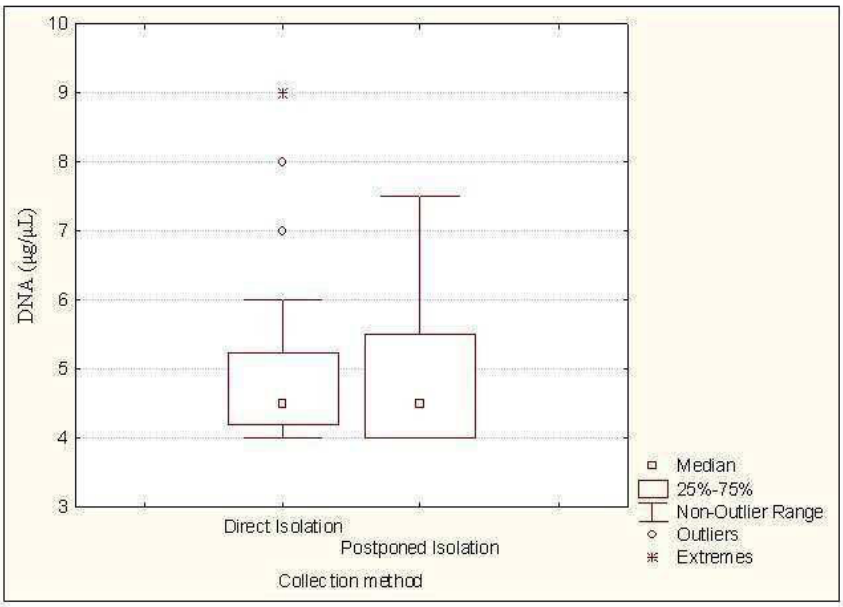

Direct Isolation (Live Isolation)
Stems 41
Stems 41
Stems 41
Stems 41
Stems 41
Control
240
3900
2600
1300
0
234
234
234
236
Postponed Isolation (Post-frozen Isolation)
Stems 41
Stems 41
Stems 41
Stems 41
Stems 41
Control
240
234
236
234

Fig. 2


10
9
8
7
6
5
4
3
DNA (µg/µL)
Direct Isolation
Postponed Isolation
Collection method
Median
25%-75%
Non-Outlier Range
Outliers
Extremes