

## Comparison of four methods of extracting DNA from *D. gallinae* (Acari: Dermanyssidae)

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**Abstract** – *Dermanyssus gallinae* is one of the most serious ectoparasites of poultry and it has been implicated as a vector of several major pathogenic diseases. Molecular detection of such pathogens in mites is crucial and therefore, an important step is the extraction of their DNA from mites. So, we compared four DNA extraction protocols from engorged and unfed individual mites: a conventional method using a Cethyl Trimethyl Ammonium Bromide buffer (CTAB), a Chelex resin, a Qiasm DNA extraction kit and a more recent one filter-based technology (FTA). The DNA samples have been tested for their ability to be amplified by an amplification of a *D. gallinae* 16S rRNA gene region. The best results were obtained using CTAB and Qiagen methods at the same time with unfed and engorged mites (96% and 100% of amplified samples). FTA produced similar results when using unfed mites but not when processing engorged ones (96% and 70%). Finally, the Chelex method was the least efficient in terms of DNA amplification, especially when applied on engorged individuals (50%). The possible inhibitor role of these Chelex extracted DNA was demonstrated by the means of a PCR control on PUC plasmid. No difference was observed with CTAB, Qiasm DNA extraction kit or FTA methods using DNA extracted one year before.

### *D. gallinae* / DNA extraction / engorged mites

## 1. INTRODUCTION

The chicken mite or poultry red mite, *Dermanyssus gallinae* (DeGeer, 1875) is a cosmopolitan hematophagous obligate ectoparasite of wild and domestic birds. It is the most economically important ectoparasite of layer hens causing debilitation, decreased egg production and anaemia [7, 15]. Furthermore, *D. gallinae* like other Dermanyssoidea have been involved as vectors of several major diseases (for review [27]). Among human pathogenic agents, *D. gallinae* has been demonstrated

to be an experimental vector of *Coxiella burnetii*, the bacteria responsible for Q fever [30]. Durden et al. [9] and Chamberlain and Sikes [6] have also proven that *D. gallinae* is able to transmit the eastern encephalitis virus by biting during blood feeding. This parasite has also been implicated in the vectorial transmission of animal epidemic agents such as the fowlpox virus or the Newcastle disease virus (NDV) where transovarian transmission was demonstrated [2, 23].

To detect pathogens in vectors, molecular methods are useful tools that may be further developed. In Acari, these techniques have been mainly applied on

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species of economic and medical importance such as ticks, scabies mites and crop pests [5, 13, 14, 16, 29]. Concerning the superfamily of Dermanysoidea, the Laelapidae and Varroidae families have been the most studied from a molecular standpoint but these mites are not hematophagous whereas the presence of blood in *D. gallinae* could entail some additional difficulties [22].

In addition to the detection of viruses or bacteria in poultry red mites, these molecular methods would also lead to progress in the knowledge of their genetics: population genetics, gene transmission, pseudo arrhenotoky, mechanisms that are involved in the development of acaricide resistance [3, 18]. All these studies require DNA and therefore the development of an efficient extraction protocol. In this study, we compared four DNA extraction protocols based on different principles, from unfed and engorged adult *D. gallinae* individuals. We tested the ability of these DNA extracts to be reliably amplified by a specific PCR amplification and then the same ability after one year of storage.

## 2. MATERIALS AND METHODS

### 2.1. Mite samples

Mites were collected from different poultry farms, in the Drôme, Ain, and Ardèche regions (Rhône-Alpes, France). Individuals were kept in 70% ethanol and frozen at  $-20^{\circ}\text{C}$ , or directly frozen at  $-20^{\circ}\text{C}$ .

### 2.2. Mite DNA extraction methods

For each DNA extraction method, 60 mites (30 unfed and 30 engorged) were tested, except in the case of DNA extracted one year before where sample sizes ranged from 22 to 60 specimens. Extractions were

performed on individual mites, frozen as described. The concentration of each DNA sample was determined by OD 260 and OD 280 measurements, with a biophotometer (Eppendorf, Hamburg, Germany) and the purity was evaluated by the ratio OD260/OD280.

#### 2.2.1. *Cethyl Trimethyl Ammonium Bromide (CTAB) method*

Individual mites were crushed with a sterile plastic pestle in a 1.5 mL microcentrifuge tube bottom containing 200  $\mu\text{L}$  of extraction buffer, (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 0.2% 2-mercaptoethanol), preheated to  $65^{\circ}\text{C}$ . This homogenate was incubated at  $65^{\circ}\text{C}$  for 1 h. Proteins were removed with one volume of chloroform/isoamyl alcohol. DNA was then precipitated from the aqueous layer by adding one volume of isopropanol, letting the sample stand for 2 or 3 h at  $-20^{\circ}\text{C}$  and spun at 18 000 g for 30 min. The DNA pellet was then washed with 300  $\mu\text{L}$  of ethanol 70%, dried for 10 to 15 min at  $50^{\circ}\text{C}$ , and resuspended in 20  $\mu\text{L}$  of ultrapure water. Tubes were placed for 2 h at  $4^{\circ}\text{C}$  and DNA were resuspended again before being stored at  $-20^{\circ}\text{C}$ .

#### 2.2.2. *Chelex method*

A single *D. gallinae* mite was crushed using a P200 micropipette tip, in a microtube containing 40  $\mu\text{L}$  of 5% Chelex resin (Instagene Matrix, Biorad, Hercules, CA, USA). Suspensions were vigorously vortexed for 10 s, incubated at  $56^{\circ}\text{C}$  for 35 min, vortexed again for 10 s, and incubated at  $95^{\circ}\text{C}$  for 15 min. Suspensions were then vortexed and centrifuged at 15 300 g for 3 min to allow easy removal of the DNA solution from the top of the tube, with care taken to avoid removing any Chelex resin from the bottom. This DNA solution was collected in a 0.5 mL

tube, to be stored at  $-20\text{ }^{\circ}\text{C}$  until PCR reaction.

### 2.2.3. Qiagen DNA extraction kit

DNA extraction was performed using the Qiamp DNA extraction kit (Qiagen, Hilden, Germany) according to the tissue protocol, with some adjustments due to the weight of one *D. gallinae*, about 11 mg, compared to the recommended tissue weight of 25 mg: briefly, mites were crushed in 20 to 25  $\mu\text{L}$  PBS, and volume was adjusted to 80 to 100  $\mu\text{L}$  with ATL buffer. Other reactive volumes were also modified (10  $\mu\text{L}$  proteinase K, 100  $\mu\text{L}$  ATL buffer, 100  $\mu\text{L}$  ethanol, 260  $\mu\text{L}$  AW1 and AW2 buffer). The step with proteinase K was prolonged for at least 5 h, at  $56\text{ }^{\circ}\text{C}$ . Genetic material was finally eluted from the column with 50  $\mu\text{L}$  of AE buffer.

### 2.2.4. Filter-based technology (FTA) method

A single *D. gallinae* mite was crushed against the microcentrifuge tube wall, using a P200 micropipette tip, and 20  $\mu\text{L}$  PBS pH 7.2 were added. This mix was homogenized by pipetting, and 5  $\mu\text{L}$  was carefully applied on the FTA card, (Whatman, Middlesex, UK) allowed to air dry for 2 h, and stored at room temperature. Disks measuring 1.2-mm were punched from the FTA card (Harris, 1.2-mm micropunch, Whatman) and placed in 1.5 mL microtubes. Disks were washed twice with 100  $\mu\text{L}$  of  $\text{TE}^{-1}$  (10 mM Tris; 0.1 mM EDTA) for 3 min, and air dried on a  $56\text{ }^{\circ}\text{C}$  heating block for 10 min. Disks corresponding to engorged mites were first washed twice with FTA purification reagent (Whatman) according to the manufacturer's instructions, before being rinsed with the  $\text{TE}^{-1}$ . Disks were allowed to dry at  $56\text{ }^{\circ}\text{C}$  for 10 min, and then used directly as templates for a PCR amplification.

### 2.3. Detection of DNA from *D. gallinae* by Polymerase Chain Reaction

The efficiency of DNA extraction methods was evaluated by amplifying the mitochondrial 16S rRNA gene of *D. gallinae*, using specific primers designed for its 16S rDNA sequence (accession number L34326), F16 (5'TGGGTGCTAAGA-GAATGGATG3') and R16 (5'CCGGTCT-GAACTCAGATCAAG3'), which amplify a 377 bp region [4]. PCR reactions were performed in 25  $\mu\text{L}$  volume containing 0.8  $\mu\text{M}$  of each oligonucleotide primer, 100  $\mu\text{M}$  of each dNTP (Amersham, BioScience, Buckinghamshire, UK), 2 mM of  $\text{MgCl}_2$  (Invitrogen, Carlsbad, CA, USA), 2.5  $\mu\text{L}$  10 $\times$  Buffer (Invitrogen) and 1U of recombinant Taq polymerase (Invitrogen), and 3  $\mu\text{L}$  DNA extract, or the FTA filter disk. PCR amplifications were performed in a MWG thermal cyclor (Biotech, Ebersberg, Germany), with a cycling program consisting of a 10 min denaturation step at  $94\text{ }^{\circ}\text{C}$  followed by 35 cycles of denaturation (1 min,  $94\text{ }^{\circ}\text{C}$ ), annealing (45 s,  $55\text{ }^{\circ}\text{C}$ ) and extension (1 min 30 s,  $72\text{ }^{\circ}\text{C}$ ), and a final extension step of 10 min at  $72\text{ }^{\circ}\text{C}$ . PCR products were separated by a 2% agarose gel electrophoresis containing ethidium bromide, and DNA fragments were visualized under UV light. After a first amplification, each negative DNA sample was submitted to a second one, and the results were scored.

### 2.4. Test of an inhibitor role

A control PCR assay using M13 primers to amplify DNA from PUC plasmid was used to confirm the potential inhibitor role of some mite DNA suspensions. PCR reactions were performed in 25  $\mu\text{L}$ , adding 100 fg of PUC DNA template, (quantity previously determined as being close to the detection limit), according to the protocol described in a cloning kit (pCR II-TOPO

**Table I.** Number of amplified samples by PCR/total number of samples extracted from adult *Dermatophagoides gallinae* by one method and submitted to the amplification (%).

	CTAB method	Qiamp kit	Chelex resin	FTA
Unfed <i>D. gallinae</i> adults	30/30 (100)	30/30 (100)	25/30 (83)	29/30 (96)
Engorged <i>D. gallinae</i> adults	28/30 (93)	30/30 (100)	15/30 (50)	21/30 (70)
Total sample	58/60 (96)	60/60 (100)	40/60 (67)	50/60 (83)

TA Cloning kit, Invitrogen). Each of the negative samples in specific amplification of 16S rDNA of *D. gallinae* was added in the same proportion as in this *D. gallinae* specific amplification, i.e. 3  $\mu\text{L}$ , to the PUC amplification mix. A positive control reaction containing 3  $\mu\text{L}$  of a positive DNA-Qiagen extract was performed. We also tested the addition of 0.8  $\mu\text{g}/\mu\text{L}$  of Bovine Serum Albumin (BSA) to this latest amplification mix, containing negative samples, in order to reduce the inhibitory effect of these samples.

## 2.5. Statistical analysis

Chi-square analysis was used to study the difference between methods and between engorgement status.  $P < 0.05$  was regarded as significant.

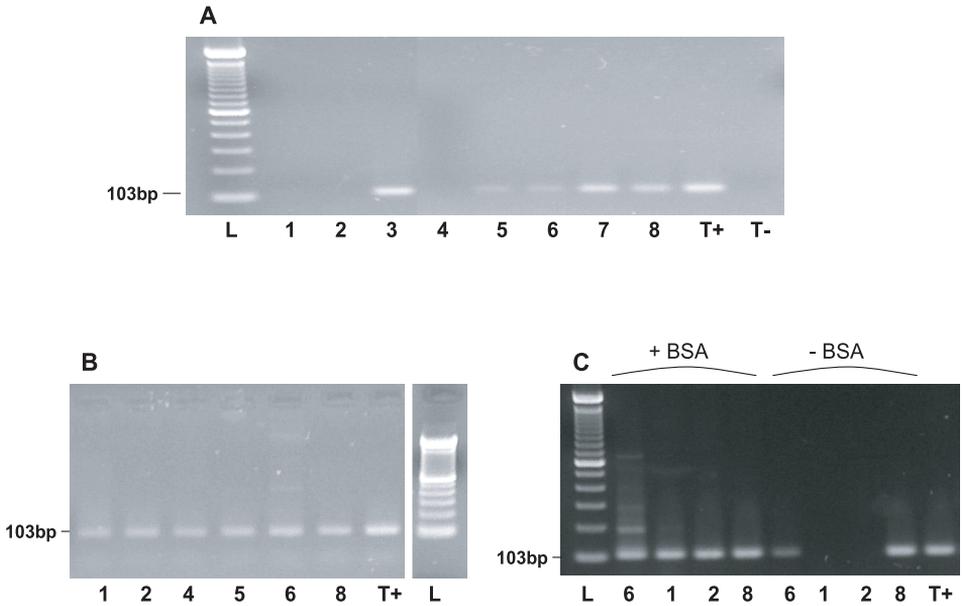
## 3. RESULTS

The concentrations of DNA extracted with the Qiamp DNA extraction kit and CTAB were very similar with averages of 20.5  $\text{ng}/\mu\text{L}$  and 43.5  $\text{ng}/\mu\text{L}$  respectively. With the Chelex resin, the average concentration was 370  $\text{ng}/\mu\text{L}$ , but the average DO260/DO280 ratio of 1.3 indicated a high level of non-genetic materials in these extracts. It was confirmed by the fact that no DNA signal was observed on an agarose gel.

We compared the efficiency of the four extraction methods, defined as being their ability to produce DNA of a sufficient quality to be amplified by a PCR (Tab. I). Without considering the engorgement status of

mites, two methods, the CTAB and Qiamp DNA extraction kit, showed a better efficiency with 96% and 100% of DNA samples being amplified by PCR. These results were significantly different from those obtained with the FTA technique (83%) and Chelex resin (67%) ( $P < 0.05$ ). If we consider only samples from unfed mites, the Chelex technique remained significantly less efficient than the others ( $P < 0.05$ ). When comparing the four methods applied to engorged mites, the CTAB and Qiamp DNA extraction kit are still the most efficient methods. The engorgement was the cause of a decrease in efficiency of 33% and 26% respectively for the Chelex and FTA methods. In order to confirm the ability of such samples to inhibit amplification, 20 negative Chelex-DNA extracts for *D. gallinae* amplification were added to the amplification mix with PUC DNA. Twelve modified the PUC amplification signal: 6 lowered this signal and 6 totally switched off this signal. To remove the putative inhibitory substances, 8 of the 12 negative Chelex suspensions were extracted a second time with the most successful DNA extraction method, a Qiamp DNA extraction kit, as described above. These re-extracted samples were then added to the M13 PCR control, and the detection signal of PUC DNA was restored for each amplification reaction. The addition of BSA to five negative Chelex extracts has also restored the PUC DNA amplification (Fig. 1).

Compared to freshly extracted DNA samples, DNA which have been extracted one year before did not show any significant difference in their ability to be amplified (100% for Qiamp DNA



**Figure 1.** Test of the inhibitor role of negative DNA-Chelex extracts on M13 amplification of PUC. (A) Modification of PUC amplification by addition of some negative DNA-Chelex extracts. Lanes 1 to 7: the 103-bp M13 region of PUC was amplified from 100 fg of plasmid DNA, in the presence of 7 different negative DNA-Chelex extracts for *D. gallinae* specific amplification. Lane 8: the same PCR product, with positive DNA-Qiagen extract for *D. gallinae* specific amplification. T+: positive reaction control containing 100 fg of PUC DNA template. T-: negative control, with water as the template. L: 100 bp ladder (Invitrogen). (B) Effect of a Qiagen extraction on DNA-Chelex extracts which inhibited the PUC amplification (samples 1, 2, 4, 5, 6 from A). PCR product of the M13 region of PUC, when these Qiagen extractions were added to the mix. (C) Effect of BSA on the inhibitory effect of DNA-Chelex extracts on the PUC amplification (samples 6, 1, 2 from A). BSA was added at 0.8  $\mu\text{g}/\mu\text{L}$  to the PUC amplification mix, in addition to negative DNA-Chelex extracts.

extraction kit, 100% for CTAB and 93% for FTA).

#### 4. DISCUSSION

According to extraction methods already published for various acari (Eriophyidae, Ixodidae, Laelapidae, Phytoseiidae, Tetranychidae, Trombididae), we chose and compared three conventional methods for extracting DNA from *D. gallinae*: a CTAB method, with a lysis step followed by a phenol chloroform precipitation [17, 20], a Qiamp DNA extraction kit

using separation with a column [8, 11], and a Chelex resin [10, 14, 21, 25, 28, 29]. Another attractive method, the FTA technology, was also tested, and compared to the others. It has been reported for detection of pathogenic DNA [19] and particularly for the detection of microsporidia in fire ants and of *Francisella tularensis* in pools of ticks, with satisfying results [12, 24].

The CTAB and Qiamp DNA extraction kit protocols gave the best results in our studies with an efficiency close to 100%, regardless of the engorgement status of the mites, while with the Chelex method, only 67% of efficiency in amplification

was obtained. These results were correlated with the concentration and the purity for each method: Chelex samples seemed to be rich in DNA, but their OD260nm/OD280nm ratio indicated an important contamination by non genetic material, whereas concentrations of CTAB and Qiamp DNA extraction kit samples were lower, but with a ratio showing that DNA was pure. When applied to engorged individuals, the Chelex method gave DNA successfully amplified in only 50% of the cases. This suggests that the presence of blood, combined with a very simple extraction procedure, may interfere with PCR amplification and this possible inhibition was assessed in two ways. First, amplification of PUC plasmid DNA with M13 primers was regularly inhibited when some negative Chelex samples were added to this control PCR mix. In addition, the inhibitory effect of these samples was overcome, by extracting them a second time with the Qiamp DNA extraction kit, or by using BSA, known for reducing the inhibitory effect of haemoglobin [1]. However, the authors of studies using this Chelex resin did not notice any difficulties but they worked on non hematophagous mites (Tetranychidae and Phytoseiidae) [14, 21, 25, 29]. Some authors have already noted that DNA template extracted by Chelex may not be sufficiently purified for standard PCR amplification [13]. Moreover, the blood presence in our specimens, known to be a PCR inhibitor, may have increased this problem [26]. Schwartz et al. [22] demonstrated that inhibitors of PCR amplification were present in engorged ticks and they recommend the use of a sufficiently rigorous extraction step to remove any potential PCR inhibitors.

The FTA technology applied to unfed mites obtained results similar to those with CTAB and the Qiamp DNA extraction kit. Decreasing efficiency was noted when working on blood-fed specimens, while the

protocol recommended for samples containing blood was used. This technique could be improved when applying it to blood-fed mites. For example, an additional purification step prior to application of the mite suspension onto the FTA card may be required, e.g. centrifugation to remove larger debris, in order to overcome diminished sensitivity.

One essential point in studies concerning arthropods is the research of pathogenic agents to assess their potential vectorial role. Detection of pathogens inside mites may be carried out on blood-fed specimens, artificially engorged or taken from breeding facilities. The Qiamp DNA extraction kit and CTAB methods are shown by the present study to be the most adapted but are time consuming. FTA technology gives less satisfying results than do conventional methods (CTAB and Qiamp DNA extraction kit), but is rapid to perform, less expensive and does not require laboratory equipment, at least for collecting DNA samples. In spite of the loss of 15% in efficiency, the FTA method could be interesting and would be the most suitable when it is necessary to analyse a large number of samples taken from breeding facilities, since it permits the collection of DNA samples *in situ* and facilitates their storage and shipment prior to laboratory analysis. Finally, we would not recommend the Chelex method in order to detect pathogen DNA in mites, since our experiments demonstrated that samples extracted with this method contain PCR inhibitors, which could yield an underestimate of the presence of DNA pathogens.

We also observed that each of these methods is able to preserve DNA samples at room temperature for FTA and at  $-20^{\circ}\text{C}$  for the three others. During this study, we also noticed with the CTAB method, an improvement in amplification results when mite samples were preserved in alcohol and stored at  $-20^{\circ}\text{C}$ , prior to DNA extraction, instead of being directly stored

at  $-20\text{ }^{\circ}\text{C}$  (data not shown). Therefore, we recommend preserving specimens in alcohol, and then at  $-20\text{ }^{\circ}\text{C}$  before DNA extraction.

We succeeded in extracting DNA from a single *D. gallinae* mite using different methods. Thus, the method of choice will be directed by the sample size and whether the mites contain blood or not. Since extraction efficiency is not always 100%, this described PCR amplification would also be useful for a positive control of DNA extraction before searching for target pathogen DNA by other specific PCR or molecular markers, especially if no amplification signal is detected.

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