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A rapid protocol for clearing, staining, and mounting of Arthropoda: Trombiculidae, Pediculidae and Pulicidae

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Abstract. This is a rapid protocol proposed after its prolonged usage for the clearing, staining, and mounting of the medically important hematophagous ectoparasites obtained from human and small mammals collected in Tamil Nadu and Kerala, India. During the biodiversity study on ectoparasites, this altered protocol improved significantly the visibility of the major essential taxonomic key characters present on the minute body of the uncharacterized chigger mites, fleas, and lice viewed under phase-contrast microscopy for identification. This modified method will be enormously useful for imparting training, teaching, and research on acarology.

Key words: ectoparasites, clearing, staining, mounting, Arthropoda.

Introduction

Arthropoda, the dominant animal kingdom includes insects, spiders, ticks, mites, and other joint-leg animals. Insects only include nearly 55% of all species of animals in the world (Barrow Clough 1992). Very few arthropods act as hematophagous ectoparasites, feed body fluids/blood of the hosts, and act as vectors for the transmission of different vector-borne diseases like scrub typhus and plague. Rodents were anesthetized for the collection of various ectoparasites and the ectoparasites were harvested from the captured rodents. The ectoparasites collected from these animals were preserved in 80% ethanol, transferred to a clearing agent and mounted in Hoyer's medium, examined under a microscope, and identified up to species level. Since there are many studies initiated to study the ectoparasites, identification of ectoparasites, preserving the specimens of ectoparasites is very important nowadays for confirming the appropriate vectors. A cost-effective saline mount method was performed to identify the scabies mites from skin scrapings (Kandi 2017). Clearing, staining, and mounting the ectoparasitic specimens ensures prolonged usage, and a new method for improving the visibility and longevity of the specimens is of paramount importance. A modified mounting technique using KOH revealed the different life stages of flea (Kandi 2018). Clearing media for the well-sclerotized specimens could make the tiny and soft-bodied specimens, such as chigger mites, lice, and others to transparent (Faraji & Bakker 2008).

Many mounting media like Canada balsam, gum chloral media such as Hoyers or Berlese (Chick 2016), gum chloral hydrate, Faure's modification of Berlese (Chick 2011) but procuring chloral hydrate became problematic (Chick 2010). Thus it was replaced by gum Arabic and lactic acid as a clearing agent (Dioni 2003). Iodine was used as a stain in the gram staining method long back during 1884 (Gurr 1952). Many stains like lignin pink (Hugh 1973), acid fuchsin (Evans et al. 1961), modification of Dioni's mounting media (Chick 2018) and rose bengal (Krantz & Walter 2009) were used but Faraji and Bakker 2008 included Iodine in the mounting media to act as the stain.

In general, arthropod insects are less than 1 mm in length which should be mounted on glass microscope slides. Normally, collected specimens for slide mounting need to be cleared, stained, and dehydrated before placement in the final mounting medium, mounted properly by using special chemicals for prolonged use. Numerous chemicals were used to date, both as clearing agents and mounting agents (Krantz 1978). Various protocols and chemical agents were used for clearing, staining, and mounting ectoparasites. We have recently developed rapid protocol/methods for clearing, staining, and mounting different ectoparasites. We found some difficulties in using standard clearing agents as per the older available protocols to preserve the various specimens like chigger mites, fleas, and lice. To avoid this difficulty, a new protocol for clearing, staining, and mounting hematophagous ectoparasites is described here.

Materials and Methods

Chigger mites, fleas, and lice are small, and proper identification is only possible after mounting specimens on microscope slides. The following various chemicals have been employed both as clearing agents and as mountants. The mounting medium significantly enhances the image and clarity of the fine structures of ectoparasites under phase-contrast microscopy. All ectoparasite specimens were preserved on the slide and also deposited in Mosquito / Ectoparasites Museum, Entomology Laboratory of ICMR-Vector Control Research Centre Field Station, Madurai, Tamil Nadu. Here, we first describe the chemicals required, the procedure for clearing and mounting, and then illustrate the usefulness of this method.

Materials required for the preparation of rapid ectoparasites clearing solution

Chigger mites

(1.) 50% Lactic Acid
(2.) 25% Phenol crystal
(3.) 0.5% Iodine resublimed p.a. (Faraji 2008)
(4.) 25% Distilled H_2O

Lice and fleas

(1.) 20% Potassium Hydroxide
(2.) Concentrated Hydrochloric acid
(3.) Dehydration (Ethanol: 50%, 70% & 90%)
(4.) Lacto-phenol solution (Ratio:2:1:1)

The aforementioned chemicals are used for the preparation of a clearing solution for chigger mites, lice, and fleas.

Ectoparasites mounting media

(1.) 100% of Rapid permanent Hoyer's medium (Lewis 1954)

(2.) 0.5% Iodine resublimed p.a. (Faraji 2008)

All the chemicals are prepared and filtered in a muslin cloth 3-5 times; the mounting media is ready to use when there are no small air bubbles left in the mixture (1-2 days). This process of clearing, mounting, and sealing should be undertaken in a laboratory with a good ventilation facility.

Chigger mites clearing and staining protocol

Preserve the chigger mite specimens in 70% ethanol. Take a cavity slide. One drop of rapid chigger mite clearing solution is added to the center of the slide. Place the chigger mites' specimen using a brush inside a cavity slide. Place the cavity side inside a hot air oven kept at 60-70 ºC for 10-15 minutes. After cooling, these chigger mites' specimens are ready to mount. Mount the chigger mites in the rapid permanent mountant Hoyer's medium. A minimum quantity of mountant was placed on the glass slide to prevent it from spreading outside and keep the coverslip over it. Dry the mounted preparation on a hot plate or inside a hot air oven at 80-90 °C for ½ hour. Afterward, the excess mountant should be cleaned by rubbing with a wet white cotton bud to clean the coverslip edges. Then the sealing process of the edge of the coverslip was undertaken using a small camel hair brush by applying a transparent nail polish (Disney 1983). Finally, the slides are ready to be placed inside the slide box. Finally, identification, labeling, and preservation will follow suit.

After clearing and mounting, the mite specimen is hard to see and missed in this process. To overcome this difficulty, Iodine is added as one of the ingredients in the preparation of the clearing solution. This has significantly enhanced the image and clarity of these minuscule structures of chigger mites.

Lice and fleas clearing protocol

Preserve the lice and flea specimens in 70% ethanol. Take a petri dish. Using a fine brush put the flea or lice specimen inside the small petri dish with 5 ml of rapid clearing agent one (20% Potassium hydroxide) and place this inside a hot air oven (80-90 ºC) for one hour. After cooling, they were transferred to water containing a few drops of concentrated Hydrochloric acid and kept for 5-10 minutes which was followed by the dehydration process in alcohol by ascending grades / 10 minutes in each 40%, 70%, and 90% concentrations. Afterward, 5 ml of rapid Clearing agent two prepared in lactophenol solution was taken in a small petri dish and place the fleas or lice inside this solution for 2-3 minutes at room temperature using a fine brush. Now the fleas or lice specimens are ready to be mounted. Mount the fleas or lice in the rapid mountant Hoyer's medium. A minimum quantity of mountant was placed on the cavity glass slide to prevent it from spreading outside and keep the coverslip over it. Dry the mounted preparation on a hot plate or inside a hot air oven at 70-80 °C for ½ hour. Afterward, the excess mountant should be cleaned by rubbing with a wet white cotton bud to clean the coverslip edges. Then the sealing process of the edge of the coverslip was undertaken using a small camel hair brush by smoothly applying a transparent nail polish (Disney 1983). Finally, the slides are ready to be placed inside the slide box which will be followed by identification, labeling, and preservation and finally loaded inside a slide cabinet (Brown 1998).

Photographic observation

The microscope with the ZEISS Axio camera MRC (60-c1"1,0X) was used in this study. Images were taken under phase contrast (using objective: 5x, lamb filter no 4 and 5, condenser mode no 2). By this method, images were combined to produce very sharp images of morphological and chaetotaxy characters.

Results

All these images of the trombiculid chigger mites, lice, and fleas enhance the visibility of these insects (Fig. 1-3). There is very good clarity obtained for viewing the minute morphological and chaetotaxy of ectoparasites. Besides that, there is enhanced visibility of the important key characters required for taxonomic identification up to the species level. In the case of chigger mites, there is clear cut sharpness in the images obtained to show the morphology of scutum shape with all setae, idiosomal setal arrangements cheliceral blade and palps present in the Gnathosomal region, and leg segmentation with setae which are very much required for the taxonomic identification of trombiculid vector chigger mites *Leptotrombidium deliense* and *Schoengastiella ligula* (Fig. 1b,

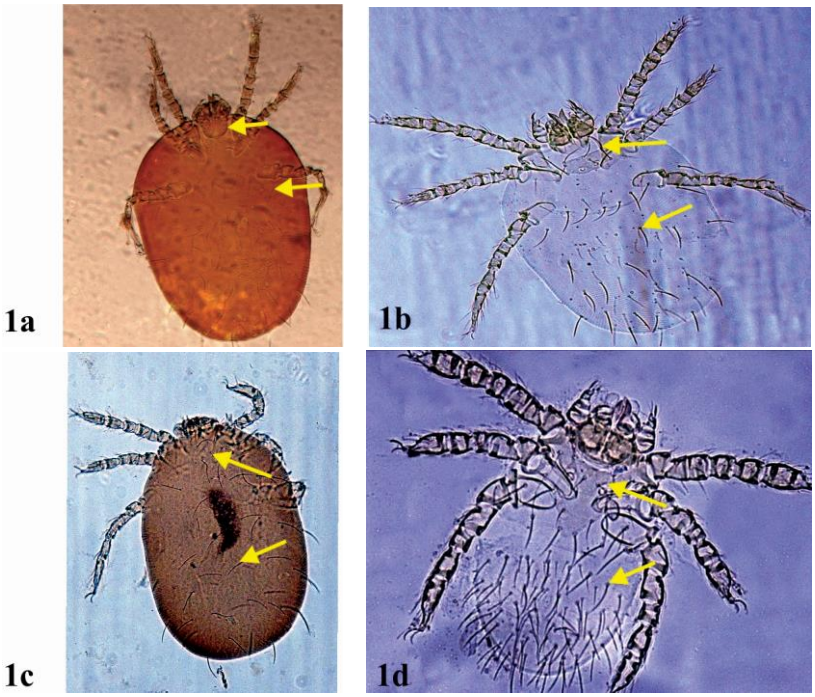

Figure 1. Chigger mites (Larva): 1a,c Normal - dorsal view, scutum shape and setae; Idiosoma setal arrangement; 1b,d Modified protocol - dorsal view, scutum shape and setae; Idiosoma setal arrangement; 1a, b.-*Leptotrombidium deliense*; 1c,d -*Schoengastiella ligula*.

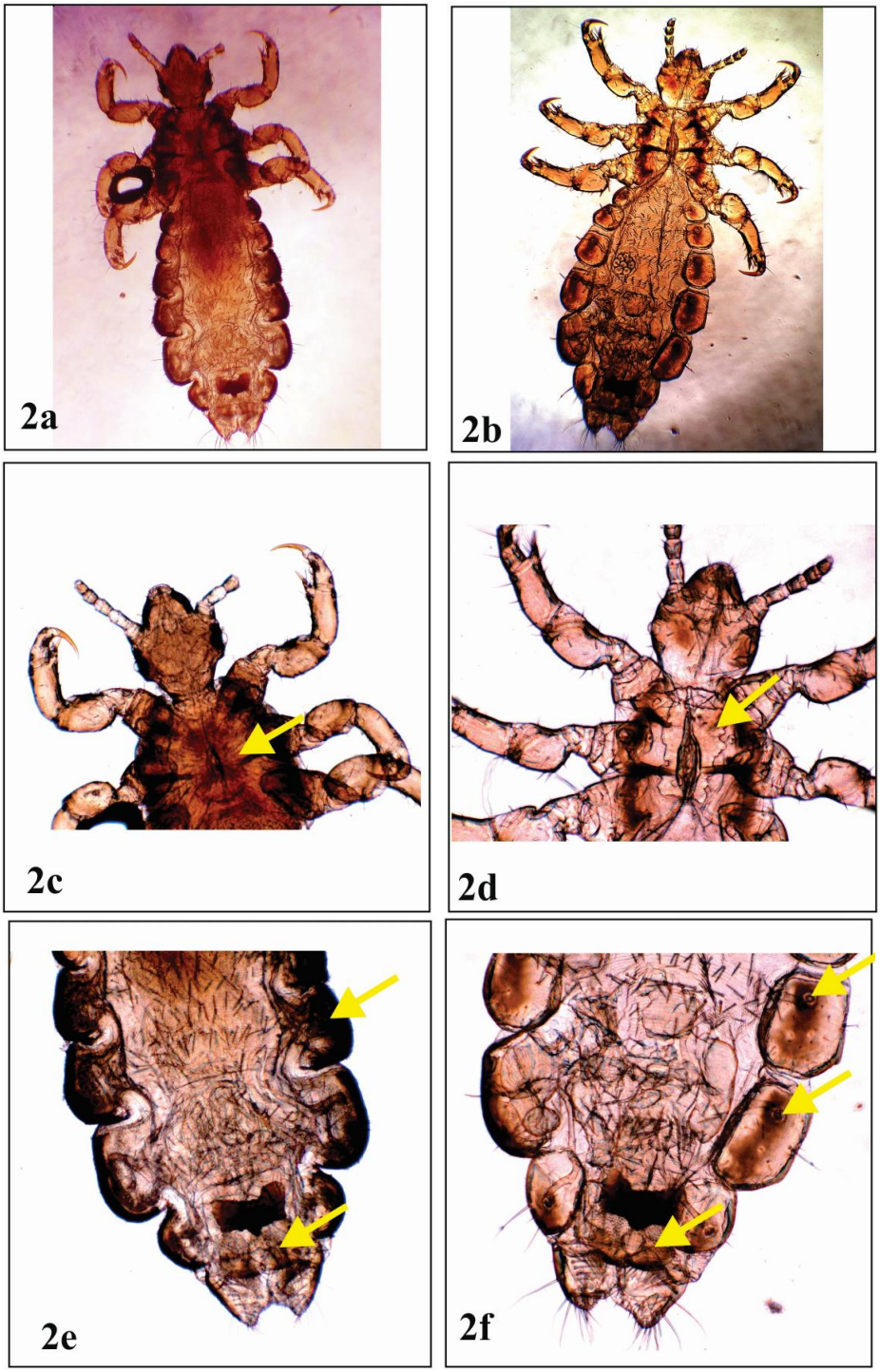

Figure 2. Louse (♀-*Pediculus sp.*): 2a Normal - dorsal view; 2b Modified protocol - dorsal view; 2c, 2e Normal - thoracic sternal plate; abdominal spiracle, genital lope & others; 2d, 2f Modified protocol - thoraxic sternal plate; abdominal spiracle, genital lope & other setal characters.

1d). At the same time, the normal slides showed no clear cut distinction of various body parts as observed in the modified protocol (Fig 1a, 1c). By this method, family Pediculidae species of lice characters were visible. Thoracic sternal plate, abdominal spiracle, and genital lobe were visible by this method (Fig. 2b, 2d, 2f). But these characters were not well defined and demarcated markedly in the normal protocol slides (Fig. 2a, 2c, 2e). This rapid method of processing helped to identify the species belonging to the family Pu- licidae. Many taxonomical key identification characters like sternite lower portion of genitalia shape, setal arrangements, and spermathecae at the basal portion of the tail were seen by this modified method (Fig. 3b, 3d, 3f, 3h). At the same time, the slides prepared by the normal method lacked these significant variations (Fig. 3a, 3c, 3e, 3g).

Discussion

Chigger mites, fleas, and lice are very small, and proper

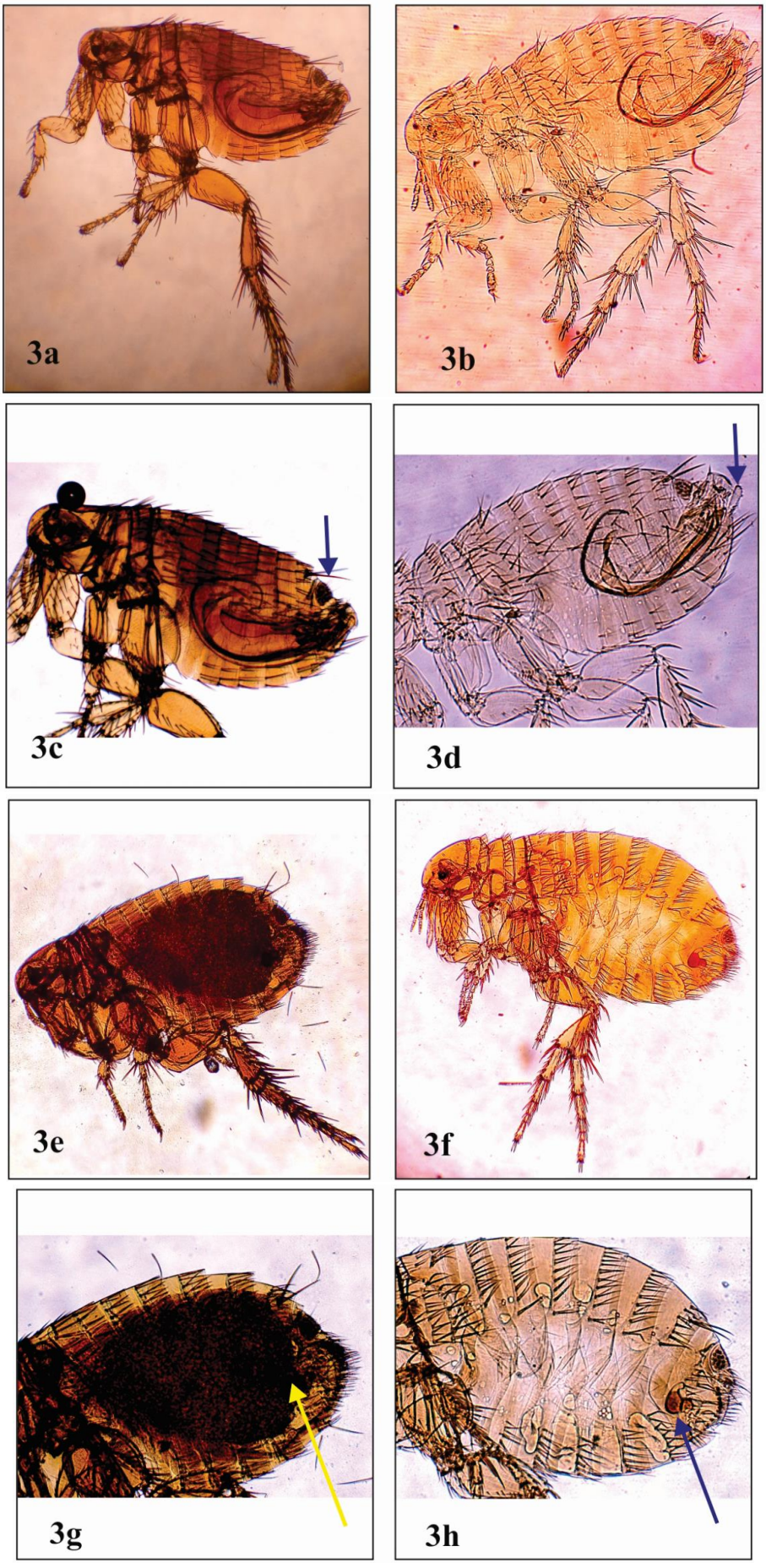

Figure 3. Fleas: 3a-d ♂ *Xenopsylla cheopis*; 3a,c Normal - dorsal view; sternite lower portion of genitalia shape and setal arrangement; 3b,d Modified protocol - dorsal view; sternite lower portion of genitalia and setal arrangement; 3e–h ♀ *Xenopsylla astia;* 3e,f Normal - dorsal view; spermathecae at the basal portion of a tail; 3f,h Modified protocol - dorsal view; spermathecae at the basal portion of the tail.

identifications are only possible after mounting those specimens on microscope slides. Many chemicals are employed both as clearing agents and as mountants (Faraji & Bakker 2008). Caustic potash (Potassium hydroxide) is an inorganic, colorless, solid chemical, it makes the exoskeleton more transparent, and helps in dissolving unwanted internal tissues present in the body of these insects. Here clearing and staining of chigger mite slides was done using lactophenol and Iodine kept at 60-700 which drastically reduced the clearing duration compared to an earlier study (Faraji 2008). Similarly, the clearance and staining procedure for lice and fleas was modified for the first time using 2 clearing agents which rapid the clarity, sharpness and greatly reduced the processing duration compared to other studies (Palma 1978, Campbell 2018). This proposed mounting medium significantly enhanced the image and clarity of the fine structures of ectoparasites in phase-contrast microscopy. In our laboratory, we used one agent for clearing and one medium for mounting. This unique and efficient method described in this manuscript is the outcome of our untiring efforts to describe thousands of ectoparasites belonging to different groups collected, mounted, processed, and identified from two different states of India viz. Tamil Nadu and Kerala. The clearing method practiced here takes a very short duration compared to all the previous reported studies (Table 1). Here staining of these animal chigger mites, lice, and fleas rapid the visibility (Iodine) as practiced earlier only in plant mites (Faraji 2008). This study described different species of ectoparasite comprised of different Families namely Trombiculidae, Pediculidae, and Pulicidae prevalent from these two states. Such slides were ringed with nail varnish or enamel paint (Disney 1983). These microscopic slides prepared for our studies were long-lasting permanent slides and, showed good clarity for the different minute structures without any damage caused to the invaluable specimen after prolonged storage (Brown 1998). Different body parts samples are viewed clearly when viewed under the microscope. This can be employed as a method for educating the acarology subjects to our M.Sc. Public Health Entomology students. By this process, chigger mites, lice, and flea specimens will be properly cleared and allowed the transmitted light to pass through them. The clearing process dissolved the very minute soft body parts and allowed these morphological and chaetotaxy characters are seen very clearly and entirely once these specimens were slide-mounted. This modified protocol will be a rapid permanent medium extremely useful for mounting these specimens that are to be drawn or photographed (Lewis 1954). This standardized method of the mounting specimen is highly useful for our ongoing taxonomy and biodiversity studies on ectoparasites and also for the proposed future faunistic surveys to be undertaken shortly. Because two chemicals are used for clearing, one for staining and one for mounting, this method is very efficient if many lice and fleas belonging to different groups have to be mounted and identified. Thus this rapid, modified protocol is the easiest method developed for producing cleared, well stained, and mounted animal chigger mites, fleas, and lice specimens.

Table 1. Comparison of clearing duration of ectoparasites.

Ectoparasites	Previous clearing duration	Present clearing duration
Mites	1 hours (Faraji,2008)	15 minutes
Lice	24 hours (Palma,1978)	1 hour
Fleas	24 hours (Campbell, 2018)	1 hour

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