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Title: Assessment of cutaneous and serum oxidative stress changes in dogs infested with *Sarcoptes scabiei* var *canis*

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Ethical Endorsement

The ethics and guideline safeguarding the rights, dis-abuse, and welfare of animal subjects was ratified by the University of Ibadan's Ethical Board (UI-ACUREC-17-0027).

Conflict of interest

The authors declare no conflict of interest.

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Author's Contributions

Study was conceived by N.A. Sadiq, designed by B.O. Emikpe and materially supported by T.O. Omobowale. Sample collection, laboratory assays, statistical analysis and interpretation was done by O.C. Nwufoh. Supervision, funding and manuscript writing was done by all authors. All authors proof read and approved the final version of manuscript.

Abstract

The evaluation of oxidative and anti-oxidative activities in skin and serum of scabietic dogs was studied. 20 healthy and 5 scabietic dogs were used. Healthy dogs were grouped as group A (Control; 10 dogs) and group B (10 dogs). Group B dogs were housed with the 5 scabietic dogs.

Hydrogen peroxide (H_2O_2), Glutathione peroxidase (GPX), Glutathione-S-transferase (GST), Superoxide dismutase (SOD), Reduced glutathione (GSH), and Total protein (TP) activities were weighed in skin and serum of healthy and scabietic dogs. Malonyldialdehyde (MDA) amount was measured in skin while Myeloperoxidase (MPO) amount was measured in serum of both healthy and scabietic dogs.

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Increase and decrease in free radical generation (H_2O_2) was noted with the use of serum and skin samples while GPX and SOD amounts in skin and serum was significantly higher in scabietic dogs. GST and GSH values were significantly higher in skin sample whereas GSH amount was not altered in serum. Total protein values decreased significantly in serum but decreased insignificantly in skin of infested dogs. MDA (skin) and MPO (serum) amounts were elevated in scabietic dogs.

Results showed that skin could be alternatives or complementary care samples in evaluation of oxidant, antioxidant balance, prognosis and monitoring of scabies therapy.

Key Words: Dogs; *Sarcoptes scabiei*; Skin; Serum; Anti-oxidants; Oxidants

1. Introduction

Canine sarcopticosis exists as a communicable cutaneous ailment of dogs which began by microscopic organisms, known as mites. *Sarcoptes scabiei* var *canis* lies popularly as the most described incriminator of scabies in dogs where they interfere with their health and productivity. When overlooked, alopecia, scaling, secondary pyoderma and dried skin crusts with serum exudates are observed (Walton and Currie, 2007). Several findings have confirmed the incidence of oxidative stress in animals infested by

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parasites (Smith and Bryant, 1989). Kocyigit et al., (2005) also gave strength to these findings as inflammatory cells known as reactive oxygen species (ROS) are produced in response to inflammatory reactions and parasite burdens. The production of reactive oxygen species (ROS) has been well related to adverse skin reactions such as skin discolorations, autoimmune reaction, allergies, wrinkles, inflammation, and keratin anomalies (Bickers and Athar, 2006). In a bid to guard the host against parasite invasion, the antioxidant mechanism of the host is triggered. This prompting leads to the activation of certain enzymes to arrest the production of free radicals. However, Knight (2000) noted that an inequity arising from the excessive assembly of ROS and the failure of the subject's antioxidative mechanism in neutralizing the free radicals would alter cellular function and breed biologic damage.

The skin tunneling activity of the canine astigmatic mite makes the skin a major object of oxidative stress (Mark et al., 2015). Cross et al. (1998) also gave credence to the oxidative stress on the skin as there are generations of reactive oxygen species in the keratinocytes following the exposure of the skin to endogenous and environmental pro-oxidants. The roles of age and body condition of subjects cannot also be downplayed in the generation of reactive oxygen species on the skin. Baumann et al., (2016) reported that an advancement of age births a drop in the function of skeletal muscles as seen with the consequent loss of muscle quality and quantity. Furthermore, a continuous decline in body condition also promotes an increase in the activity of these free radicals thereby overwhelming anti-oxidation activities and establishing oxidative stress (Trouba et al., 2002).

Several other studies have extensively associated the activity of *Sarcoptes scabie* var *canis* to the presence of damaging free radicals in the serum of infested dogs (Beigh et al., 2013), Egyptian buffaloes

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(Allaam et al., 2014), and Dromedary camels (Saleh et al., 2010). However, there remains very scanty information on the oxidant and antioxidant system on sarcoptes infested skin.

In the country of study, there are no scientific reports on the subject of oxidant and antioxidant assays in the serum of dogs exposed to *Sarcoptes scabiei* var *canis* and the evaluation of such using skin biopsies has not been previously reported. Hence, the intention of the present research was to demonstrate skin sample usage as a complementary alternative in the assessment of oxidation and antioxidation in dogs infested with sarcoptic mites. Research also aimed at investigating the progressive pattern of oxidation and antioxidation on both the skin and serum of infested dogs so as to have a robust knowledge on the pathogenesis of canine scabies.

2.0 MATERIALS AND METHODS

2.1 Ethical Approval

The principal ethics regulating, supervising and justifying animal care, use, rights, safety and welfare was approved by The Animal Care, Use and Research Ethics Committee (UI-ACUREC 17-0027) of study institution. Approval of research and ethical conducts was obtained after the scientific and public health merits of the study was justified. Animal welfare factors such as feeding, shelter, handling, experimental techniques and fate (treatment) of dogs were assessed to ensure that the impact on animal well-being was devoid of suffering.

2.2 Dog recruitment and grouping

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A total of twenty (20) healthy dogs (mongrels) were recruited for study. Dogs were less than 6 months old and dogs were in good body condition as marked by absence of bone and rib prominence. Dogs were recruited from dog homes and then sheltered in the fumigated research kennels of The College of Animal Health. Dogs were subjected to environmental and clinical adjustments for two weeks before their exposure to sarcoptic mites. Five (5) sarcoptes infested dogs were also enrolled for study after their skin scrapping investigations revealed that they were solely sarcoptes infested (Soulsby, 1982). The mite free dogs were then grouped into two. Group A dogs had ten (10) healthy dogs and were assigned control dogs while Group B dogs had the other ten (10) healthy dogs. Group B dogs were then housed together with the five (5) sarcoptes exposed dogs as described by Nwufoh et al. (2018). With the successful establishment of mites, the five (5) donor dogs were immediately withdrawn and treated appropriately. All other mite exposed dogs were treated after studies.

2.3 Sample Collection

Three millimeter punch biopsy was used to obtain skin samples from lesion zones. These zones were at the axillae, elbows, limbs, dorsal and ventral aspects of the neck. Since study aimed at establishing the use of the skin in determining oxidant and antioxidant response, skin biopsies were continuously taken at the 2^{nd}, 4^{th}, 6^{th} and 8^{th} week post infestation. Skin biopsies were immediately taken into ice after collection. Blood samples from both groups A and B subjects were obtained via the cephalic vein at the 4^{th} and 8^{th} week following mite exposure. Blood was left to clot for serum yield. Skin samples were further cleaned with phosphate buffer saline (pH 7.4) and then blot dried. With the fraction of 1g damp tissue to 1.15% Tris–potassium chloride buffer (10ml), skin homogenate was prepared.

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2.4 Oxidative Stress Assays

Serum myeloperoxidase (sMPO) in the serum was assayed following Xia and Zweier's technique (1997). Serum (10 µL) was added to O-dianisidine (200 µL) and H_2O_2 mixture. The resulting mix was read at 0, 30 and 60 seconds correspondingly at 450 nm wavelengths.

The estimation of glutathione (GSH) in the skin and serum sample followed routine of Jollow et al. (1974). Serum (250 µL) was transferred to 250 µL of 4% sulfosalicylic acid contained in a laboratory tube. The tube containing the mixture was then centrifuged for 5 mins at 4000rfc. 20µL of the resulting supernatant was then plated into the 96-well microtiter plates. The wells then had Ellman's reagent (180 µL) pipetted to them. Reading of reactive absorbance was with a spectrophotometer at 405nm wavelength against the blank with distilled water. Same procedure was carried out for the skin homogenates.

Activity of glutathione peroxidase (GPX) was estimated in accordance with Buetler et al. (1963). 250µL of skin homogenate was added to reaction mixtures of 0.1M phosphate buffer (250 µL), 50µL $NaNO_3$, 100µL GSH, 100µL H_2O_2, and 300µL of distilled water. Succeeding mixture was then subjected to incubation for 5 minutes at 37˚c. 250µL of TCA was then drawn into the mix before centrifuging at 3,000 rpm for 5 minutes. 100µL of dibasic potassium phosphate with 50µL of DTNB ended up to 50µL of supernatant skin homogenates. Mixture was then aliquoted into wells of 96-well microtiter plate. Absorbance then was noted at 405 nm against the blank of distilled water. Similar technique was followed for the serum sample.

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The estimation of hydrogen peroxide (H_2O_2) generation followed the WOLFF'S (1994) technique. 0.1M phosphate buffer (100µL), $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ (50 µL) (MW= 392.14), 20 µL Sorbitol (MW= 182.2), Xanthine Oxidase (10 µL) (XO), H_2SO_4 (25 µL) and serum sample (50 µL) were all drawn into wells of the 96 well microtiter plate. The plate was then rotated rapidly and left incubating only for 30 minutes at room temperature. Reading of absorbance (490 nm) with distilled water as blank was achieved. Procedure was also repeated for skin homogenates. The H_2O_2 standard curve was used in extrapolating H_2O_2 generation.

Superoxide dismutase (SOD) level in skin homogenates and serum samples was determined as described by Omobowale et al. (2015). Serum (20 µL) was added to 0.05M carbonate buffer (250 µL). Acidified reconstituted adrenaline (300 µL) was then added to the ensuing mixture after which absorbance alterations were noted for 180s at 490nm frequency. Change in absorbance was read for every 30s. Same procedure was carried out with the skin homogenates.

The total protein calculation was demonstrated consequent upon biurets technique (Gornall et al., 1949). 96–well microtiter plates containing 100 µL of biuret reagent had 50 µL of skin homogenates. The plate stood at 20°C for just 30 minutes before its reading with the spectrophotometer at 490nm wavelength. Reading conclusions were drawn on the total protein standard curve. This was also repeated for serum samples.

Method used in the determination of Gluthathione S Transferase (GST) activity was described by Habig et al. (1974). 10 µl of skin homogenate sample was mixed with 140 µL of phosphate buffer saline.

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Following was the inclusion of 10 µl of GSH. 50 µl of CDNB (1- chloro, 2, 4 dinitrobenzol) was also added to the mixture. Half plate was first read before the other half was read at wavelength of 405nm.

The level of Malondialdehyde (MDA) was estimated as detailed by Varshney and Kale (1990). Microtiter plates had Tris-Kcl (400 µL), 30% TCA (125 µL), skin homogenate (100 µL) and 0.75% of TBA (125 µL) arranged in 0.2M Hydrochloric acid. Incubation of plate was then achieved for 45 mins at 80^{o}C in a water bath, ice cooled and centrifuged for 15 mins at 3000rfc. Absorbance was further noted at 490nm. Amount of lipid peroxidation level was estimated on a molar extinction coefficient of 1.56×10^{5}/M/cm.

2.5 Statistical Analysis

Pairwise multiple comparisons procedure (Tukey test) was used to compare means of each row with means of every other row and detected differences were considered to be significant at $P<0.05$. The statistical analysis of the data was achieved with the one-way analysis of variance (ANOVA) and the statistical package used for analysis was GraphPad Prism 6.

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3.0 RESULTS

Microscopic examination of skin scrapes revealed the presence of *Sarcoptes scabiei* var *canis* as was identified by their classic and characteristic oval, ventrally flattened and dorsally convex tortoise like body. Evidence of stout dorsal setae and several cuticular spines was also noted (Fain, 1968).

Table 1

Mean ± SD of H_2O_2, MDA, GPX, GST, SOD and GSH on the skin of dogs

Parameters	H_2O_2 µmol $mg^{-1}p$	MDA µmol/ml	GPX units mg^{-1}	GST units $mg^{-1}p$	SOD units $mg^{-1}p$	GSH µg ml^{-1}	TP g/dl
Healthy	33.91±1.91	0.22±0.09	103.91±39.14^{b}	0.02±0.01^{b}	12.32±3.01^{b}	66.29±1.13^{b}	1.94±0.99
2nd WPI	35.12±1.11	0.35±0.11	122.54±41.95^{b}	0.32±0.01^{a}	21.22±6.81^{a}	69.51±2.91^{b}	2.15±1.40

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4th WPI	34.81±1.32	0.38±0.06	166.13±57.71[b]	0.39±0.13[a]	25.31±7.21[a]	69.52±1.14[b]	1.31±0.77
6th WPI	36.13±4.42	0.38±0.14	321.82±56.82[a]	0.11±0.04[b]	30.92±5.24[a]	73.71±2.34[a]	1.18±0.67
8th WPI	31.45±1.34	0.43±0.13	302.33±63.53[a]	0.11±0.06[b]	30.55±4.61[a]	74.21±1.82[a]	0.85±0.27

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WPI: Week Post Infestation, **H_2O_2**: Hydrogen peroxide, **MDA**: Malonyldialdehyde, **GSH-Px**: Glutathione peroxidase, **GST**: Glutathione-S-transferase, **SOD**: Superoxide dismutase, **GSH**: Reduced glutathione, **TP**: Total protein.

Mean values with different superscripts [a,b,] along the same column differ statistically ($P< 0.05$).

The level of the stress marker (H_2O_2) on the skin of both infested and non-infested dogs varied from Table 1. The activity of H_2O_2 increased by the 2nd week of infestation but decreased by the 4th week. Higher amounts of hydrogen peroxide peaked by the 6th week post infestation and an obvious drop was noted by the 8th week of infestation. Cutaneous MDA level in scabies free dogs was also lower than what was obtained in infested dogs fortnightly. The skin antioxidant enzyme action (GPX) significantly increased at the 6th and 8th week of mite infestation. However, there was an insignificant reduction in the activity of skin GPX enzyme recorded on the 8th week of mite infestation. The cutaneous GST enzyme significantly increased in mite infested animals at the 2nd and 4th week of mite exposure. On the other hand, GST anti-oxidation significantly reduced at the 6th and 8th week of mite exposure. Actions of SOD enzyme was somewhat synonymous with GST activity as enzyme actions significantly increased in mite infested dogs till the 6th week of infestation. Reduced glutathione (GSH) estimates increased significantly in mite exposed dogs only on the 6th and 8th week post infestation. Total protein amounts decreased in infested animals even though decrease was not significant.

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Table 2

Mean ± SD of H_2O_2, MPO, GPX, SOD, and GSH in serum samples of dogs

Parameters	H_2O_2 µmol $mg^{-1}p$	MPO µmol/min	GPX units mg^{-1}	SOD units $mg^{-1}p$	GSH µg ml^{-1}	TP g/dl
Healthy	46.13±3.12[a]	60.81±6.63	28.11±5.01[b]	6.17±1.02[b]	70.81±9.13	6.13±1.01[a]
4th WPI	51.62±5.44[a]	67.11±17.42	39.92±2.53[a]	8.50±0.23[a]	71.24±12.22	4.41±0.16[b]
8th WPI	35.33±2.51[b]	68.83±7.93	42.01±4.13[a]	8.60±0.62[a]	70.54±0.83	4.38±0.31[b]

WPI: Week Post Infestation, **H_2O_2**: Hydrogen peroxide, **MPO**: Myeloperoxidase, **GSH-Px**: Glutathione peroxidase, **SOD**: Superoxide dismutase, **GSH**: Reduced glutathione, TP: Total protein.

Mean values with different superscripts [a,b,] along the same column differ statistically (P< 0.05)

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The serum H_2O_2 activity (Table 2) between non-infested and infested dogs differed significantly. Worthy of note here is the link between the skin H_2O_2 and serum H_2O_2 activity as there was a significant decline ($P< 0.05$) in the level of oxidation by 8^{th} week of mite infestation.

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Serum MPO actions were elevated continuously in mite exposed dogs. The serum GPX antioxidation was lower in healthy dogs as compared with infested dogs where it improved significantly till the 8th week of infestation. This same development was also observed with SOD antioxidation as the difference in activity from the 4th till 8th week post infestation did not increase significantly. Notable was the high amounts of skin GPX and SOD when compared with the amounts in the serum. There was no major difference in the levels of serum GSH in non-infested and infested dogs. The total protein values of infested animals significantly decreased from values of non-infested animals.

Discussion and Conclusion

This study describes enzymatic activities of the skin and serum of dogs exposed to *Sarcoptes scabiei* var *canis* towards the complementary health care use of skin biopsies for evaluation of oxidative and anti-oxidative activities in skin infections. From this study, elevated amounts of skin H_2O_2, serum H_2O_2 and skin MDA in sarcoptes scabies infested dogs establishes oxidative stress in *Sarcoptes scabiei* var *canis* infestation in dogs. This is the first account on oxidative stress detection with the use of skin biopsies from sarcoptes infested dogs. Study findings of oxidative stress in serum is in conformity with several other described studies on existence of oxidative stress in serum of sarcoptes infested buffaloes (Dimri et al., 2008a), Psoroptes infested sheep (Dimri et al., 2010), and Demodicosis in dogs (Dimri et al., 2008b).

Noteworthy was the corresponding decreased activity of skin H_2O_2 and serum H_2O_2 of scabies infested dogs by the 8th week of infestation. This decrease could be attributable to the immunological status of the host (Walton, 2010). The first few weeks of mite exposure comes with an increase in mite numbers, mite activities

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and consequently an increase in generation of free radicals. Since dogs used in this study were healthy and were not immunosuppressed to achieve infestation, the immune response could have improved to bring about a gradual decrease in mite number and toxic metabolites. The pattern of decrease in the production of free radicals (H_2O_2) observed with skin samples is similar to that of serum thus establishing that oxidative stress observed in serum depicts that on the skin. However, the percentage of increase in H_2O_2 amounts was higher in the serum despite the predominance of mite activities on the skin. This most probably suggests that the serum remains the best sample for the detection of oxidative stress in canine sarcopticosis as the activities of *Sarcoptes scabiei* var *canis* increases inflammatory cell exudation in the serum (Bernabucci et al., 2005). Evidence of inflammatory response to scabies was also confirmed with the increase in serum levels of MPO in sarcoptes infested dogs.

The part of lipid peroxidation (LPO) as a recognized pathway of cellular damage in the pathogenesis of scabies in dogs (Camkerten et al., 2009) was also backed with the result of skin MDA elevation all through study weeks. This outcome on the skin of dogs is similar to that of Dimri et al., (2010) where skin levels of LPO in psoroptes infested sheep significantly differed from healthy sheep.

The antioxidant defense system was at its best in scavenging free radicals. The skin GPX levels increased significantly and then finally crowned at the 6^{th} week of infestation. The decrease in the cutaneous GPX antioxidation at the 8^{th} week post infestation followed the same trend as the skin and serum H_2O_2 oxidation activity. This pattern of cutaneous antioxidation remains the first account of such even as it contradicts several other reports of serum antioxidant defense exhaustion during generation of free radicals (Singh et al., 2011). Findings of increase in enzymatic antioxidation from this study may perhaps point to the possibility of the

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defense system of the subjects remaining at its prime since healthy animals were induced for study. However, GPX scavenging was stronger in the skin than the serum as detected amounts of the enzyme from skin samples were very much higher than serum levels. Even though it may seem quite laborious to obtain skin biopsies in comparison to obtaining blood samples, the skin seems more of the best approach in determining GPX antioxidation activity. The fraction of increase in GPX levels on the skin also gives strength to the preference of the skin for GPX assessments. The abundance of GPX enzyme on the skin may likely be in response to the damage resulting from the mite's tunneling actions and the presence of fecal and egg remnants in the tunnels. These activities tend to increase the itching on the skin thereby endangering the skin to oxidative injuries of which the GPX enzyme guards against.

It is well documented that the presence of SOD aids the generation of O_2 from reactive oxygen species (Fang et al., 2002). Results of study thereby shows an abundance of SOD activities on the skin till the 8th week of infestation while GST affairs on the skin declined by the 6th week of infestation. The SOD pattern of sustained activity all through study weeks was evident with the use of both skin and serum samples but the results of the assay with the skin biopsy highlighted increased amounts of the enzyme on the skin. SOD activity in skin infections can thus be better detected with the use of skin samples.

Reduced glutathione (GSH) activity on the skin improved till the 6th week of infestation. However, no significant variance between estimates at the 6th and 8th week of infestation was observed. Although there was no important alteration in the serum and skin GSH levels, the ratio of increase in skin GSH levels presents the skin as fit for consideration in the determination of GSH amounts. Total protein amounts in the skin and serum were observed lower in infested animals. This finding backs up earlier findings of decreased total

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protein amounts in mangy dogs (Nwufoh et al., 2019) as the mites continuously sucks up fluid and predisposes host to exudative dermatitis.

Since dogs used for study were less than six (6) months old, age would not have contributed to the cutaneous oxidative stress observed as skeletal muscles of such age brackets remain firm in strength and integrity. However, the compromise in muscle strength, and quality often associated with oxidative stress (Baumann et al., 2016) could have contributed to oxidation observed from study as activities of *Sarcoptes scabiei* are largely predominant on the skin. The persistent itch that results during sarcoptic mite infestation could gradually deplete muscle quality and integrity thereby birthing an increase in reactive oxygen species.

The short study duration of only 8 weeks could possibly be linked to the failure of the antioxidant defense mechanism to shut down in subjects as well. Most probably, the oxidation and anti-oxidation outcomes of chronic and progressive cases of scabies could possibly be different as this phase is characterized by millions of mites (Crusted scabies) and abundance of mite activities. Bearing this in mind, an overwhelming invasion of *Sarcoptes scabiei* mites would lead to a larger assembly of reactive oxygen species (ROS) and accordingly result to a poor antioxidant defense system.

The breed of dogs exposed to this study may perhaps have contributed to the vibrancy of the antioxidant defense mechanism. Mongrels were used for all studies and this breed of dogs seems resilient and hence is mostly used for hunting in country of study.

In conclusion, activities of toxic radicals are incriminated in the bi-weekly pathogenesis of sarcoptic mange in dogs but oxidative damage may account for the severity observed in earlier stages. The outcomes of study could be very useful in drawing out strategic approaches to the treatment plan of canine scabies. Skin biopsies

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may be alternative and complementary health care samples for canine scabies diagnosis, prognosis, and elucidation of antioxidant activities especially in GPX, SOD and GSH assays. Serum samples can best be preferred for H_2O_2 determination alongside diagnosis and monitoring of scabies therapy.

Conflict of interest

The authors declare no conflict of interest.

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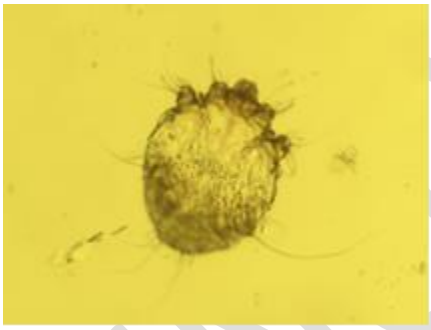

Fig 1: Sarcoptes scabiei var canis isolated from some sampled dogs (10% potassium hydroxide preparation, X100 magnification).

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