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Short communication

Pre- and post-therapy circulating immuno-stimulatory and immuno-suppressive cytokines in dogs with juvenile-onset generalized demodecosis

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ABSTRACT

Overproliferation of *Demodex* mites in dogs with compromised immunity attributed to the development of canine demodecosis. Whether clinical signs of canine demodecosis are triggered by genetically-mediated specific immunodeficiency in dogs or the *Demodex* mites induce lesions in hair follicles and result in compromised immunity is yet to be fully explored. To unravel the concealments of immunosuppression in canine demodecosis the present study was aimed to estimate the levels of circulating cytokines, pre- and post-therapy in nine dogs with juvenile-onset generalized demodecosis. At day 60 post-therapy of recommended amitraz rinse, significant ($p \leq 0.02$) reduction in circulating IL-10 level was observed compared to its level before the start of the therapy (day 0). However, significant alterations in circulating levels of TNF-α and IFN-γ were not observed in these dogs at day 60 post-therapy as compared to their day 0 levels. A strong positive correlation between circulating level of IL-10 and mites population was observed both on day 0 ($r^2 = 0.656$; $p \leq 0.005$) and day 60 post-therapy ($r^2 = 0.575$; $p \leq 0.018$). Therefore, our findings suggest that *Demodex* mites induce immunosuppression in dogs during clinical disease and mites burden seems to be responsible for the development of generalized demodecosis.

1. Introduction

Although overproliferation of *Demodex* mites in immuno-compromised dogs occurs regularly, it is far from common, at least in the studies published so far (Singh and Dimri, 2014). In many studies evaluating larger numbers of dogs with hyperadrenocorticism or dogs with neoplastic disease on chemotherapy, only very few if any of those dogs develop demodecosis. Recently, it has been proposed that *Demodex* mites are the normal cutaneous parasites in most of the healthy dogs (Ravera et al., 2013) and these mites interplay with both the innate and adaptive immune systems of the host to evade host immunity and produce clinical disease in dogs (Singh et al., 2010; Félix et al., 2013; Kumari et al., 2017, 2018). Canine demodecosis is differentiated into localized versus generalized forms. Localized demodecosis has a good prognosis, and the majority of the cases spontaneously recover without any miticidal treatment (Scott et al., 2001). But, generalized demodecosis could be a grave dermatological condition in dogs if not managed well (Miller et al., 2013). Therefore, therapeutic management of canine generalized demodecosis remains a major challenge to veterinary dermatologists. The exact pathogenesis of generalized canine demodecosis is yet to be well-established; however, an aberration in immune status is thought to be one of the most significant factors apart from the invading organism (De Bosschere et al., 2007; Singh et al., 2010, 2011). Cytokines secretion from T lymphocytes plays important role in the immune response of dogs against mites-induced dermatological disorders including demodecosis (Félix et al., 2013; Singh and Dimri, 2014; Singh et al., 2014). The progression of the disease from localized to generalized form in dogs is greatly influenced by the breach in T-cell immunity and overproduction of immunosuppressive cytokines (Caswell et al., 1997; Singh et al., 2010; Félix et al., 2013; Kumari et al., 2017). Increased TGF-β mRNA expression might be a key factor for revealing differences between the mechanisms of the onset of

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localized and generalized demodecosis (Tani et al., 2002). A strong association of elevated IL-10 levels in the blood of dogs with recurrent demodecosis (Félix et al., 2013) and dogs with generalized demodecosis has recently been demonstrated (Kumari et al., 2017). Clinical cure of demodecosis is associated with parasiticidal treatment and the resultant reduction in the number of *Demodex* mites (Forton, 2012; Miller et al., 2013). Despite a lot of data on the pathogenesis of demodecosis in dogs, yet it is an open debate whether *Demodex* mites are responsible for inducing immunosuppression in the host for their perpetuation, or these mites perpetuate in previously immunosuppressed dogs? Evidences suggest that the host immunity plays an important role in the control of *Demodex* proliferation in canine skin (Singh et al., 2010; Félix et al., 2013; Kumari et al., 2017), however, the precise information and mechanism of immune response as well as host–mite affiliation in canine demodecosis is yet to be convincingly established (Kumari et al., 2018; Souza et al., 2018). Concealment of immunosuppression in canine demodecosis can be elucidated by evaluating the immune status of dogs before the start of miticidal therapy and comparing it with the immune status of the same dogs after completion of the miticidal therapy. Studies on establishing the correlation of immuno-stimulatory and immuno-suppressive cytokines with mites burden would further add to our existing knowledge about the pathogenesis of demodecosis especially immuno-pathogenesis of canine demodecosis. Therefore, the present study was undertaken to evaluate the immune status of dogs suffering from generalized demodecosis by estimating the circulating immuno-regulatory cytokines before and after the miticidal therapy.

2. Material and methods

Client-owned dogs presented for diagnosis and treatment of dermatological ailments at Teaching Veterinary Clinical Complex (Kothari Hospital) of the University were examined. Diagnosis of demodecosis was made based on detection of the adult stages and immature stages of *Demodex* mites in deep skin scrapes obtained from the skin lesions of affected dogs. Dogs detected positive for presence of *Demodex* mites in skin scraps and having either five minimum affected skin areas ($> 10\,cm^2$ each) or a single-affected body skin region ($> 100\ cm^2$) or having at least one affected paw (pododemodecosis) were considered to be suffering from generalized demodecosis (Paterson et al., 2009). The dogs with generalized demodecosis, which were not treated with any ectoparasiticide or steroidal anti-inflammatory drugs in the last 30 days before the clinical examination, were enrolled in the present study. All the dogs included in this study were more than four months old, and had the history of the start of the clinical disease before attaining 18-months-age and hence considered to have juvenile-onset of canine demodecosis. All these dogs also had the history of routine deworming and vaccination, and were free from any other concurrent systemic and/or infectious disease. All the included demodecosed dogs were free from other ectoparasites infestations except for *Demodex* mites. The participating dogs also had all the physiological parameters such as body temperature, respiratory-rate and heart-rate within the normal reference range. The detection of neutrophils and intracellular cocci in the impression smears obtained from the lesional skin was used to make a diagnosis of concurrent secondary pyoderma.

The severity of *Demodex*-induced skin lesions was recorded based on four clinical signs, namely- extent of erythema, scales/crusts, comedones/papules/pustules and alopecia, and rated on a scale from 0 (absent) to 6 (extremely severe) with a maximum total score of 24 at one site. All the four scores were summed up for each affected area of the body and expressed as *Demodex*-induced skin lesions score (DSLS) for each affected site (Paterson et al., 2009). The mean of DSLS at different sites of affected areas was calculated and used for assessment of the clinical recovery in each of the diseased dogs. Per cent clinical recovery was calculated by using the formula - % clinical recovery = [(Day 0 DSLS – post-therapy date DSLS) / Day 0 DSLS] X 100. Mites count was performed in skin scrapings obtained from two different affected skin sites of one square centimetre each. The same sites were sampled on each subsequent examination. For taking skin scrapes, the selected $1\ cm^2$ area was squeezed by holding the area of skin between thumb and forefinger. Then, with the help of a blunt knife, a deep skin scrap was taken until blood oozed out. The scraped sample was transferred into a glass test tube. To this tube, 2–3 ml of 10% potassium hydroxide (KOH) solution was added and the tube was subjected to mild heating over sprit lamp until we observed for 2 bumps in the KOH solution. The tube was allowed to stand for a few minutes at room temperature. After cooling, it was centrifuged at 2500 rpm for 5 min. The supernatant was discarded and the pellet obtained was used for counting mites under the light microscope using 10X objective lens. Absolute count of different life cycle stages of mites was recorded as per the method suggested by Paterson et al. (2009).

Dogs suffering from generalized demodecosis were treated with 0.0375% solution v/v of amitraz rinse at weekly interval for eight weeks. Before each application of amitraz rinse, demodecosed dogs were given thorough bathing with 2.5% benzoyl peroxide shampoo and towel dried. Demodecosed dogs diagnosed with concurrent pyoderma were first given lincomycin injection at the dose rate of 20 mg kg body weight once a day by intramuscular route for five days and first amitraz rinse was applied on the sixth day. All the diseased dogs included in the study were clinically examined at 30 ± 03 days interval post-therapy for clinical recovery and parasitological load. Moreover, approximately 3 mL blood sample each was collected from the saphenous vein of demodecosed dogs into tubes containing clot activator on day 0 (before the start of the therapy) and 60 days (± 03) post-therapy. Serum was harvested from each blood sample, transferred into cryovials and was stored at −20 °C until the estimation of the circulating levels of cytokines.

Circulatory levels of interleukin-10 (IL-10), tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) were estimated in sera samples at day 0 and day 60 post-therapy by using canine-specific ELISA kits (RAB0524-IL-10; RAB0526-TNF-α; RAB0523-IFN-γ, Sigma-Aldrich, USA) following the experimental procedures described by the manufacturers. Levels of IL-10 and IFN-γ were expressed as ng/mL, while TNF-α level was expressed as pg/mL. The intra-assay and inter-assay reproducibility coefficients of variation of all the assayed cytokines were < 10% and < 12%, respectively.

Statistical differences between the pre- and post-therapy samples were determined by paired *t*-test using GraphPad Prism 8 software (San Diego, CA, USA). Further, Pearson's r-correlation statistics were used to determine the degree of relationship between IL-10, TNF-α and IFN-γ values, and absolute mites count on day 0 and day 60 post-therapy. Data are presented as mean ± standard error (± SE) and P values less than 0.05 were considered significant.

3. Results and discussion

Nine dogs of different breeds, identified to have the juvenile onset of generalized demodecosis, were included in the present study. Out of the nine demodecosed dogs, four dogs were found to have concurrent secondary pyoderma as well. On day 60 post-therapy, demodecosed dogs revealed significant ($p \leq 0.02$) reduction in the circulating level of IL-10 compared to the level on day 0 (Table 1). Similarly, compared to the pre-treatment values, marked decrease (29.73%) in serum level of TNF-α was also observed on day 60 post-therapy; but the decrease in TNF-α level was not statistically significant. However, there was no remarkable or significant alteration in the circulating level of IFN-γ (Table 1). A perusal of the data in Table 1 further revealed that the mite count was also significantly ($p \leq 0.001$) reduced on day 60 post-therapy compared to the count on day 0. A strong positive correlation between the circulating level of IL-10 and mites population was also observed on both day 0 ($r^2 = 0.656$; $p \leq 0.005$) and day 60 post-therapy ($r^2 = 0.575$; $p \leq 0.018$). But, there was no significant correlation between the circulating levels of TNF-α and IFN-γ, and the mites

Table 1
Circulating immuno-stimulatory and immuno-suppressive cytokines contents in dogs with juvenile-onset generalized demodecosis.

Estimated panels	Pre-therapy (Day 0)	Post-therapy (Day 60)	P values
Interleukin-10 (ng/mL)	28.87 ± 8.76	**7.33 ± 0.75**[a] (74.61%)	P = 0.0263
Tumour necrosis factor-α (pg/mL)	153.05 ± 27.54	107.55 ± 9.87 (29.73%)	P = 0.1394
Interferon-γ (ng/mL)	3.83 ± 0.08	3.84 ± 0.09	P = 0.9391
Mite counts	333.78 ± 71.04	**56.22 ± 9.86**[a](82.27%)	P = 0.0014

-Data in parenthesis indicate % reduction compared to day 0 values.
[a] Significantly differs when compared with day 0 values.

population on any of these two days of clinical examination.

Reduction in mites count on days 30 and 60 post-therapy with amitraz rinse was 52.42 ± 3.8 and 82.27 ± 1.7 per cent, respectively. However, none of the demodecosed dogs treated with amitraz rinse was detected to be completely free from mites even on day 60 post-therapy. These dogs required 120–150 days to become completely negative for the presence of any mite in skin scrapings during the recommended regimen of amitraz rinse application. Per cent improvement in DSLS of the demodecosed dogs treated with amitraz rinse was found to be 44.31 ± 3.23 and 86.29 ± 2.88 on days 30 and 60 post-therapy, respectively.

It is believed that *Demodex* mites are a normal inhabitant of canine skin and most of the dogs have a small population of these mites in their skin (Ravera et al., 2013), but the clinical manifestation of demodecosis is restricted to some dogs only. Clinical signs are triggered by genetically-mediated specific immunodeficiency that results in excessive proliferation of mites (Mason et al., 1996; It et al., 2010), or immunodeficiency is induced by the action of mites in hair follicles (Barriga et al., 1992; Ferrer et al., 2014; Kumari et al., 2017, 2018). Dogs with generalized demodecosis suffer from T-cell exhaustion, which is usually characterized by low production of supportive/stimulatory cytokines and high levels of suppressive cytokines (Kumari et al., 2017). Cytokines play an important role in immune defence against infections and their production is genetically, epigenetically, and post-transcriptionally controlled. Cytokines signalling cascade ultimately determine the effect of each cytokine on the balance between health and disease (Ritcher et al., 2018). Absolute quantity of any cytokine is proportionally related to its effect (Ritcher et al., 2018).

Our finding indicates that *Demodex* mites have induced the production of this immunosuppressive cytokine during demodecosis as after treatment with a miticide, there was not only significant decrease in the number of mites but also significant decreases in the level of IL-10 was observed. In our earlier study also, a significantly higher level of IL-10 in dogs suffering from generalized and localized demodecosis compared to healthy dogs was observed and based on that we had proposed that development of clinical demodecosis is associated with overproduction of immunosuppressive cytokines (Kumari et al., 2017). An elevated level of TNF-α in demodecosed dogs having concurrent pyoderma while no such elevation in the circulating content of TNF-α in demodecosed dogs having no concurrent pyoderma has been reported (Kumari et al., 2017). Therefore, marked, but an insignificant, reduction in TNF-α level in demodecosed dogs on day 60 post-therapy observed in the present study could be due to the clinical recovery from pyoderma in four of the demodecosed dogs having concurrent pyoderma included in this group.

Findings of the present study support the previously proposed hypothesis of cutaneous immunosuppression that may be a consequence, instead of the primary trigger, for mite overpopulation and the development of clinical demodecosis (Barriga et al., 1992; Félix et al., 2013; Kumari et al., 2017). However, these studies have not unequivocally shown this hypothesis. For validating this hypothesis one would need to have the IL-10 concentrations before the development of the disease, which none of the cited studies has evaluated. Another possibility is that some puppies initially have a higher IL-10 concentration. This may normalise in some dogs as the immune system matures, but is sufficiently high for sufficient time in some other dogs to allow a proliferation of the mites with subsequent development of a vicious cycle where mites further immunosuppress an already immunosuppressed dog. Affected dogs initially may have different Toll-like receptor expression initially that also contributes to the beginning of the disease and feeds into that vicious cycle. We have shown in a different study, that Toll-like receptor expression is different in dogs with demodecosis and normal dogs (Kumari et al., 2018). Moreover, some dogs with demodecosis self-cure speaks against a sole induction of immunosuppression by mite proliferation. Therefore, we need to clarify this issue by conducting longitudinal studies in dog litters predisposed to demodecosis to see, to which degree those changes are present before and to which degree the mites cause them. In the present study, we also observed a strong and significant positive correlation between the immunosuppressive cytokine (IL-10) and the population of mites on pre- and post-therapy examinations. Thus, *Demodex* mites proliferation seems to play a significant role in the induction of immunosuppression in dogs and the development of generalized demodecosis. On the contrary, some of the researchers have recently demonstrated that there is no marked association between IL-10 and development of clinical demodecosis in dogs (Gasparetto et al., 2018; Souza et al., 2018) as the concentrations of cytokines, chemokines and immunoglobulins could not be correlated with severity of demodecosis in dogs (Souza et al., 2018). The findings of the present study could have been differed from these studies because of differences in the clinical presentation, severity and chronicity of the disease, presence or absence of secondary infections, immunoassay techniques used, and statistical analysis. All these variables may be responsible for inconsistent results and thus difficult to compare as well.

Demodex mites seem to contribute to immunosuppression and therefore the development of generalized demodecosis. It may not be unreasonable to suggest further that the adjunct therapy using immunostimulatory agents along with miticidal agents will be more effective in the treatment of generalized demodecosis in dogs compared to use of miticides alone. However, further studies in a large number of animals with demodecosis are warranted to support our finding that *Demodex* mites can induce immunosuppression in the host for its perpetuation.

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References

Barriga, O.O., Al-Khalidi, N.W., Martin, S., Wyman, M., 1992. Evidence of immunosuppression by *Demodex canis*. Vet Immunol immunop. 32, 37–46.
Caswell, J.L., Yager, J.A., Parker, W.M., Moore, P.F., 1997. A prospective study of the immunophenotype and temporal changes in the histologic lesions of canine demodecosis. Vet. Pathol. 34, 279–287.
De Bosschere, H., Casaer, J., Neukermans, A., Baert, K., Ceulemans, T., Tavernier, P., Roels, S., 2007. Severe alopecia due to demodicosis in roe deer (*Capreolus capreolus*) in Belgium. Vet. J. 174, 665–668.

Félix, A.O.C., Guiot, E.G., Stein, M., Felix, S.R., Silva, E.F., Nobre, M.O., 2013. Comparison of systemic interleukin 10 concentrations in healthy dogs and those suffering from recurring and first time *Demodex canis* infestations. Vet. Parasitol. 193, 312–315.

Ferrer, L., Ravera, I., Silbermayr, K., 2014. Immunology and pathogenesis of canine demodicosis. Vet. Dermatol. 25, e427–e465.

Forton, F.M., 2012. Papulopustular rosacea, skin immunity and *Demodex*: pityriasis follicularum as a missing link. J. Eur. Acad. Dermatol. Venereol. 26 (1), 19–28.

Gasparetto, N.D., Almeida, A.D.B.P.F., Nakazato, L., França, E.L., França, A.C.H., Fagundes, D.L.G., Bortolini, J., Sousa, V.R.F., 2018. Density measurement of *Demodex canis* by qPCR and analysis of serum cytokine levels in dogs with different clinical forms of demodicosis. Vet. Parasitol. 257, 1–4.

It, V., Barrientos, L., López-Gappa, J., Posik, D., Diaz, S., Golijow, C., Giovambattista, G., 2010. Association of canine juvenile generalized demodicosis with the dog leukocyte antigen system. Tissue Antigens 76, 67–70.

Kumari, P., Nigam, R., Choudhury, S., Singh, S.K., Yadav, B., Kumar, D., Garg, S.K., 2018. *Demodex canis* targets TLRs to evade host immunity and induce canine demodicosis. Parasite Immunol. 40 (3). https://doi.org/10.1111/pim.12509.

Kumari, P., Nigam, R., Singh, A., Nakade, U.P., Sharma, A., Garg, S.K., Singh, S.K., 2017. *Demodex canis* regulates cholinergic system mediated immunosuppressive pathways in canine demodicosis. Parasitology 144, 1412–1416.

Mason, I.S., Mason, K.V., Lloyd, D.H., 1996. A review of the biology of canine skin with respect to the commensals *Staphylococcus intermedius*, *Demodex canis* and *Malassezia pachydermatis*. Vet. Dermatol. 7, 119–132.

Miller, W.H., Griffi, C.E., Campbell, K.L., 2013. Small Animal Dermatology. Muller and Kirk's. Elsevier, St. Louis, pp. 304–313.

Paterson, T.E., Halliwell, R.E., Fields, P.J., Louw, M.L., Louw, J.P., Ball, G.S., Pinckey, R.D., McKibben, J.S., 2009. Treatment of canine generalized demodicosis: a blind, randomized clinical trial comparing the efficacy of Advocate (Bayer Animal Health) with ivermectin. Vet. Dermatol. 20, 447–455.

Ravera, I., Altet, L., Francino, O., Sánchez, A., Roldán, W., Villanueva, S., Bardagí, M., Ferrer, L., 2013. Small *Demodex* populations colonize most parts of the skin of healthy dogs. Vet. Dermatol. 24, 168–172.

Ritcher, K.R., Nasr, A.N., Mexas, A.M., 2018. Cytokine concentrations measured by multiplex assays in canine peripheral blood samples. Vet. Pathol. 55, 53–67.

Scott, D.W., Miller Jr., W.H., Giriffin, C.E., 2001. Canine demodicosis. In: Muller, Kirk's (Ed.), Small Animal Dermatology. W.B. Saunders, Philadelphia, pp. 457–474.

Singh, S.K., Dimri, U., 2014. The immuno-pathological conversions of canine demodicosis. Vet. Parasitol. 203, 1–5.

Singh, S.K., Dimri, U., Sharma, M.C., Sharma, B., Saxena, M., 2010. Determination of CD4+ and CD8+ T cells in the peripheral blood of dogs with demodicosis. Parasitology 137, 1921–1924.

Singh, S.K., Dimri, U., Sharma, M.C., Sharma, B., Saxena, M., Kumari, P., 2014. Assessment of the cytokine profile in peripheral blood mononuclear cells of naturally *Sarcoptes scabiei* var. *Canis* infested dogs. Vet. Parasitol. 206, 253–257.

Singh, S.K., Dimri, U., Sharma, M.C., Swarup, D., Sharma, B., Pandey, H.O., Kumari, P., 2011. The role of apoptosis in immunosuppression of dogs with demodicosis. Vet. Immunol. Immunopathol. 144, 487–492.

Souza, C.P., Schissler, J.R., Contreras, E.T., Dow, S.W., Hopkins, L.S., Coy, J.W., Rosychuk, R.A.W., Hawley, J.R., Lappin, M.R., 2018. Evaluation of immunological parameters in pit bull terrier-type dogs with juvenile onset generalized demodicosis and age-matched healthy pit bull terrier-type dogs. Vet. Dermatol. 29 (6) 482-e162.

Tani, K., Morimoto, M., Hayashi, T., Inokuma, H., Ohnishi, T., Hayashiya, S., Nomura, T., Une, S., Nakaichi, M., Taura, Y., 2002. Evaluation of cytokine messenger RNA expression in peripheral blood mononuclear cells from dogs with canine demodicosis. J. Vet. Med. Sci. 64, 513–518.