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# Detection of pathogens of veterinary importance harboured by *Stomoxys calcitrans* in South African feedlots

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ABSTRACT

Stable flies are blood feeding ectoparasites of economic, veterinary and medical importance. There is scarcity of data on pathogens harboured by stable flies occurring in South African feedlots. Hence, in this study we documented stable flies occurring in three selected South African feedlots and carried out a PCR based detection for the presence of DNA of *A. marginale*, Lumpy skin disease virus (LSDV), *Ehrlichia* spp. and *Rickettsia* spp. The flies were captured with vavoua traps, identified morphologically as *Stomoxys calcitrans* which was also supported by amplification and sequencing of both *16S r*RNA and COI genes which matched with relevant species on the NCBI database. A total of 53 fly pools were used for the detection of pathogen DNA. PCR showed the presence of DNA of *Anaplasma marginale* (22.64%, 12/53) and LSDV (15.08%; 8/53). Neither *Rickettsia* nor *Ehrlichia* spp. DNA were detected from all the sampled feedlots. In conclusion, results obtained from this study showed that *S. calcitrans* in feedlots is harbouring *A. marginale* and LSDV which suggests that they may be involved in their mechanical transmission to livestock.



## Introduction

*Stomoxys calcitrans* (Diptera: Muscidae) commonly referred to as stable fly is an obligate blood sucking ectoparasite [1,2], which is increasingly considered as economically important pest of medical and veterinary significance worldwide [1,3]. Cattle are the principal host of stable flies, however, stable flies can still infest a wide host range including horses, dogs, camels, goats, pelicans, monkeys and humans [4]. Both sexes (male and female) are blood feeders which cause irritation, loss of blood and wounds on their host [5,3]. Furthermore, stable flies are interrupted feeders and consequently, this haematophagic feeding behavior makes them a mechanical vector of a wide range of pathogens including viruses, protozoa, bacteria, and helminths [3]. Some of this pathogens includes *Anaplasma marginale* [6,7], lumpy skin disease virus [8], *Bacillus anthracis* [9] and *Habronema microstoma* [10] to mention a few.

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Mechanical transmission is of high importance as it is the most threatening "indirect effect" of the blood-feeding insects which occurs through either contamination of mouthparts or regurgitation of digestive tract contents [11,3]. Stable flies also affect the cattle industry by damaging the hides of cattle during feeding [12]. The infestation of *S. calcitrans* on livestock results losses of millions of dollars per year to the cattle industry in the United States and in other affected countries which is difficult to estimate [13,14].

Bovine anaplasmosis occurs in tropical and subtropical regions throughout the world and is caused by *Anaplasma marginale* [6]. Blood-sucking arthropods principally ticks are vectors involved in the transmission while many other hematophagous diptera including *Tabanus* spp. *Haematobia irritans* and *Stomoxys calcitrans* have been implicated [15,16].

Lumpy skin disease (LSD) is a highly contagious viral diseases of cattle [17]. This disease is a transboundary disease and has been categorized by the World Organization for Animal Health (OIE) as a notifiable disease [18]. Historically, the disease is endemic in all parts of Africa from where it has spread to Europe via the Middle East [19]. Almost all blood-sucking arthropods (mosquitoes, ticks, horse flies and stable flies) have been implicated in the transmission [20,8].

There is a great number of studies and available information on ticks and tick-borne diseases in South Africa. However, there is lack of detailed studies on the role played by stable flies in transmission of pathogens to livestock, which has resulted in little or no information on the relationship of stable flies (vector) and microbial pathogens. As a result, the current study was aimed at filling in this information gap by characterizing (morphologically and genetically) stable flies at South African feedlots and determining whether they may be harbouring the LSD virus, *Anaplasma* spp., *Erhlichia* spp. and *Rickettsia* spp. as they are pathogens of veterinary and economic importance in the country.

## Materials and methods

### *Study sites and fly sample collection*

The samples were collected from three feedlots across three provinces in South Africa in Potchefstroom (26°52'41.1" S; 27°00'21.8" E) of North West province in Sasolburg (26°54' 36.0 S; 27°48'59" E) in Free State province and Polokwane (23°47'42.914" S; 29°25'17.155" E) in Limpopo province (Fig. 1). The flies were captured using Vavoua traps (3 traps per sample site) which were placed in a feedlot for a period of 4 weeks during autumn which is a warm and rainy season in South Africa (February and March). Upon collection in each trap, the flies were preserved in 70% ethanol and taken to the laboratory for further analysis.

### *Morphological identification of stable flies*

Flies were identified using standard key according to their morphological characters [21,22] under Wild Heerbrugg M5 stereo microscope (Heerbrugg, Switzerland). Photomicrograph was taken using the Nikon AZ 100 M zoom microscope (Nikon Inc., Tokyo, Japan).

### *DNA extraction from flies*

All fly specimens were morphologically identified and grouped according to feedlots of collection. Flies were pooled prior to extraction for the detection of pathogens with 20 pools each for Potchefstroom and Sasolburg which consisted of 5 flies per pool and 13 pools for Polokwane which consisted of 2 flies per pool and one pool that consisted of 3 flies. The wings were removed in order to reduce abundant exoskeletons that may influence the enzymatic action during DNA extraction [23]. DNA was extracted using salting-out method [24]. All eluted DNA was stored −20 °C until testing.

### *Amplification of stable fly DNA by PCR*

To supplement morphological identification and characterization of captured flies, PCR targeting the *CO1* and *16S rRNA* genes were conducted to amplify *S. calcitrans* DNA with expected product size of 710 bp [25] and 300 bp [26], respectively. The reactions were carried out in final volume of 25 µl which was composed of 1.5 µl of each primer (10 µM each of Forward and Reverse), 2 µl of template DNA, 7.5 µl nuclease-free water (NFW) and 12.5 µl of Amplitaq Gold 360 PCR Master Mix (Applied Biosystems, California, USA). The PCR conditions were set as follows: initial denaturation at 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s and final elongation at 72 °C for 1 min. The above-mentioned PCR conditions were applied for amplification of *16S rRNA* with annealing temperature set at 50 °C for 30 s. All PCR products were electrophoresed on a 1% agarose gel stained with ethidium bromide and visualized under ultraviolet light. All Positive PCR products were sent for sequencing at Inqaba Biotechnical Industries (Pty) Ltd in Pretoria, South Africa.

### *Detection of pathogen DNA by PCR*

Both conventional and nested PCR assays were employed for the amplification of pathogens from fly DNA. Pathogen DNA screened from stable fly includes *Anaplasma marginale*, Lumpy skin disease virus, *Erhlichia* spp. and *Rickettsia* spp. All primer

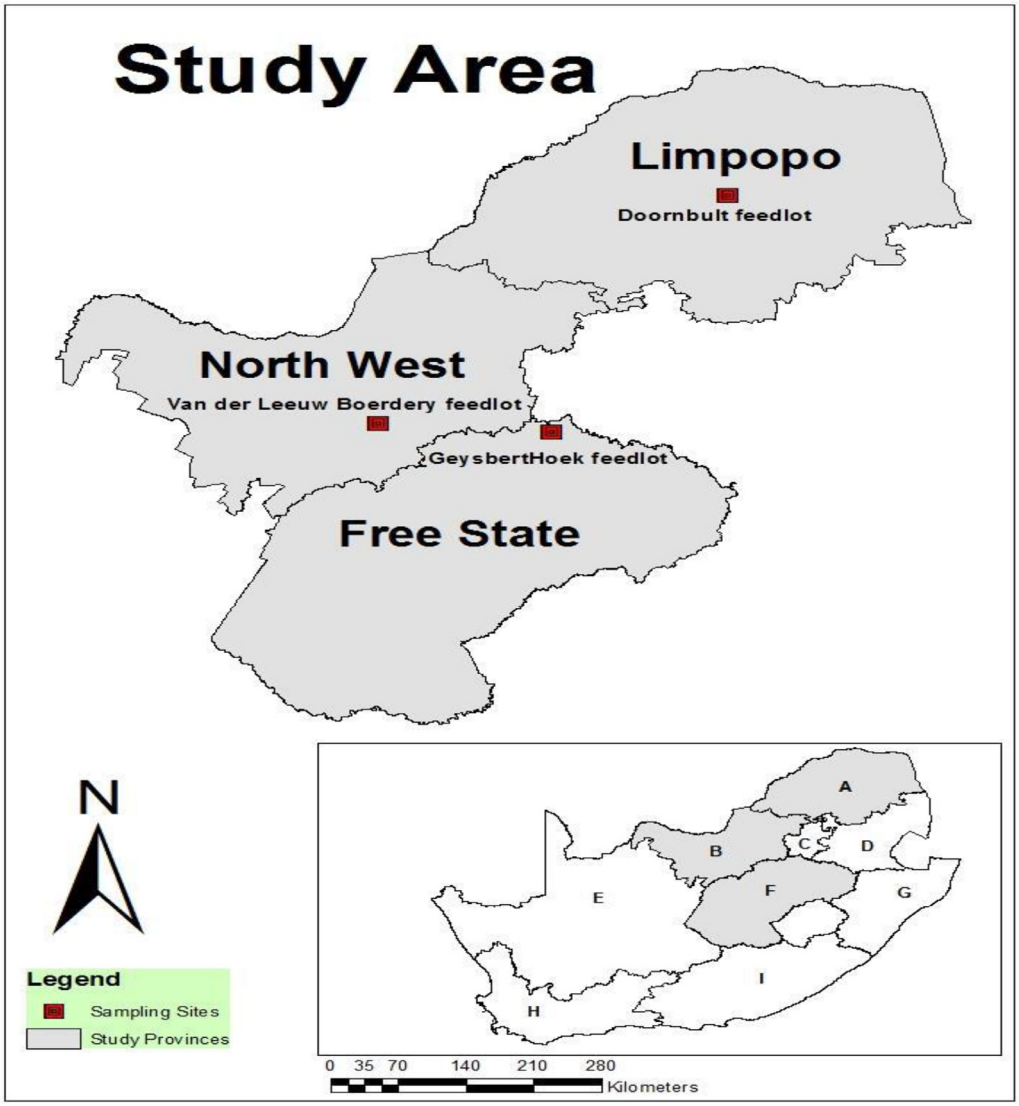


**Fig. 1.** Map of South Africa showing the three sampled provinces, namely, Limpopo **(A)** North-West **(B),** and Free State **(F).**

pairs used in the PCR assays are outlined in Table 1. The msp1$\alpha$ gene was targeted for the detection of *A. marginale* DNA from the stable flies following previously described PCR conditions [27]. For the detection of *Ehrlichia* spp. DNA, the *16S rRNA* gene was amplified with expected fragment size of 476 bp as previously described [28]. *Ehrlichia* positive DNA controls were obtained from the Center for Zoonosis Control (Hokkaido University, Japan). The genus-specific primers (Table 1) amplifying approximately 407 bp fragment of the *gltA* gene were used for the amplification of the *Rickettsia DNA*. The PCR conditions were set as previously described [29]. The primers targeting the LSDV NI-2490 fragment with a product size of 1237 bp (Table 1) were used for the amplification of Lumpy skin disease virus DNA. The PCR conditions were set as previously described [30].

## Results

*Stable flies collected from South Africa*

A total of 10195 stable files were collected from three sampled feedlots in South Africa with 9993 from Potchefstroom (North West Province), 175 from in Sasolburg (Free State Province), and 27 in Polokwane (Limpopo Province). For detection of pathogens fly pools we set as, 20 pools each for Potchefstroom and Sasolburg and 13 pools for Polokwane.

**Table 1**
Primer pairs used in the current study.

| Species | Primers | Primer sequence (5′ → 3′) | Product size (bp) | Refs. |
|---|---|---|---|---|
| Diptera | LCO1490 | GGTCAACAAATCATAAAGATATTGG | 710 | [25] |
| | HCO2198 | TAAACTTCAGGGTGACCAAAAAATCA | | |
| Diptera | N1-J-12585 | GGTCCCTTACGAATTTGAATATATCCT | 300 | [26] |
| | LR-N-12866 | ACATGATCTGAGTTCAAACCGG | | |
| *A. marginale* | msp1α-1733F | F-TGTGCTTATGGCAGACATTTCC | 1090-1113 | [27] |
| | msp1α-2957R | R-AAACCTTGTAGCCCCAACTTATCC | 1267-1290 | |
| | msp1α-3134R | R-TAACGGTCAAAACCTTTGCTTACC | | |
| *Ehrlichia* | 16S8FE | F-GGAATTCAGAGTTGGATCMTGGYTCAG | 476 | [28] |
| | B-GA1B | R-CGGGATCCCGAGTTTGCCGGGACTTCTTCT | | |
| *Rickettsia* | CS-78 F | CCAAGTATCGGTGAGGATGTA | 401 | [29] |
| | CS-323 R | GCTTCCTTAAAATTCAATAAATC | | |
| LSDV | lsd43U | GTGGAAGCCAATTAAGTAGA | 1237 | [30] |
| | lsd383U | CCCAATATTCTGCTGCTCTT | | |
| | lsd1262L | GTAAGAGGGACATTAGTTCT | | |

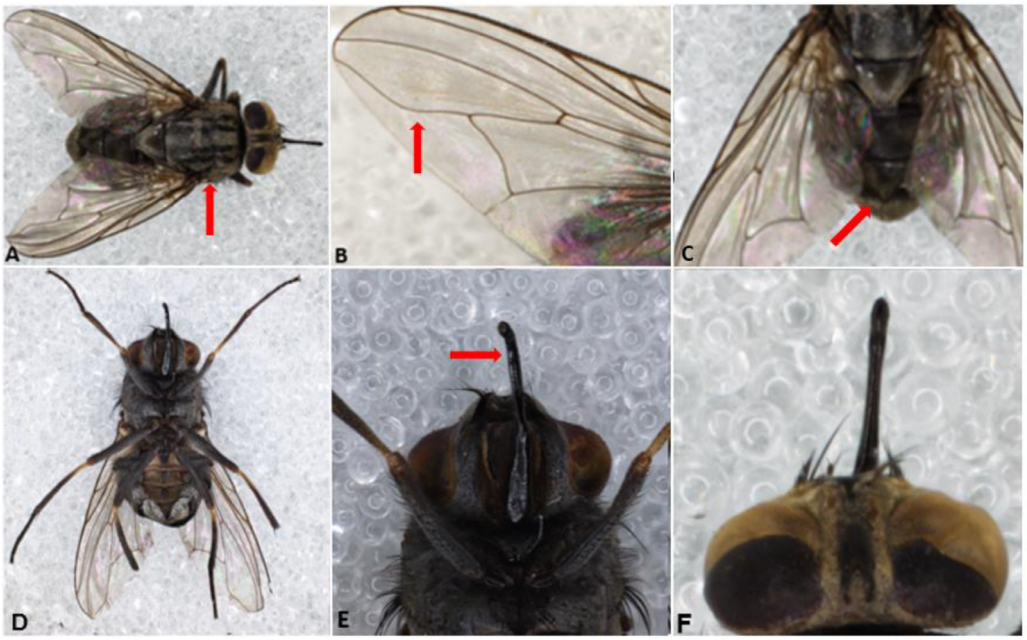


**Fig. 2.** Morphological features of stable fly (*Stomoxys calcitrans*), photos produced in the current study. **(A)** dorsal view with arrow showing checkered pattern on thorax; **(B)** wing with arrow indicating bowed fourth wing vein; **(C)** dorsal view of the abdomen with a checkered pattern; **(D)** ventral view of the whole fly; **(E)** ventral view of the Proboscis; **(F)** dorsal view of the head and proboscis.

### *Morphological identification of stable flies*

The adult stable fly measured 4 to 7 mm in body length (Fig. 2A) [21], with a grey abdomen and thorax set apart with an arrangement of patterns [21]. The thorax had four dorsal longitudinal stripes of which the two furthest stripes are shorter (Fig. 2A) [31,21]. The *S. calcitrans* was further recognized by their bowed fourth wing vein (Fig. 2B) [21], and maxillary palps which are shorter than the proboscis [32]. The abdomen had a checkered pattern, one middle spot and two parallel round spots, on the dorsal side of the second and third segment (Fig. 2C) [31]. The stable fly had a blade like proboscis (Fig. 2E and F) [32,21].

### *Molecular identification of South African stable flies*

To supplement morphological identification, molecular amplification of the *16S rRNA* and *COI* genes was carried out. BLASTn query of the *CO1* and the *16S rRNA* genes sequences had 99–100% identity with reference sequences of *S. calcitrans*

**Table 2**
Overall occurrence of pathogens in South African feedlots.

| Location | Total no. of fly sample pools | *A. marginale* (%) | *Rickettsia* (%) | *Ehrlichia* (%) | *LSDV* (%) |
|---|---|---|---|---|---|
| Potchefstroom | 100 | 16 (16.0) | 0 (0) | 0 (0) | 27 (27.0) |
| Sasolburg | 100 | 10 (10.0) | 0 (0) | 0 (0) | 0 (0) |
| Polokwane | 13 | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Total | 213 | 26 (12.21) | 0 (0) | 0 (0) | 27 (12.68) |

on the NCBI GenBank shown in supplementary file Tables S1 and S2. Sequences generated in this study have been registered in the NCBI GenBank with the accession number LC014901 and LC014902.

*Detection of pathogens from stable flies by PCR*

The DNA of *A. marginale* was amplified in 12 out of the 53 pools with an occurance of 22.6%, producing bands of appropriate size ranging between 1276 and 1290 bp targeting the msp1$\alpha$ (Supplementary Fig. S1). The occurrence of *A. marginale* was highest with 35% (7/20) in North West, followed by Free State with 25% (5/20). None of the samples ($n = 13$) collected from Limpopo province tested positive for *A. marginale* (Table 2).

The overall occurrence of LSDV was 15.0% (8/53), with the highest observation in North West 40.0% (8/20] (Table 2) (Supplementary Fig. S2). None of the samples collected from Free State ($n = 20$) and Limpopo ($n = 13$) provinces tested positive for LSDV (Table 2). The PCR produced bands with the correct length at 1237 bp. None of the samples tested were positive to neither *Rickettsia* spp. nor *Ehrlichia* spp. (Table 2).

## Discussion

In this study we collected stable flies from feedlots as they are facilities where large numbers of livestock are kept and as a result they also have large collections of manure which is good for reproduction purposes of the flies. The *S. calcitrans* was the only species recorded from fly trap collections in the present study. Morphologically *S. calcitrans* appear to be similar in size with *Musca domestica* (5–7 mm) but *S. calcitrans* are easily recognized by their moderately bent fourth wing vein, grey thorax with longitudinal stripes, distinct checkered pattern on the abdomen and a thin long proboscis that protrudes from under the head as previously described [31,21,33]. Similarly, in a study conducted by Evert, [34] in a feedlot near Heidelburg in Gauteng province of South Africa on temporal distribution and relative abundance of stable flies, only *S. calcitrans* was present. More flies were captured at feedlot in the North West province than the Limpopo and Free State provinces. We suspect the discrepancy in number flies captured could be due disturbances due to high rainfall during collection at Free State province and too hot temperatures at Limpopo province which might have resulted in less activity of the flies.

Sequenced PCR amplicons of the *CO1* and the *16S rRNA* genes of *S. calcitrans* matched with *S. calcitrans* sequences available on NCBI GenBank database with 99%–100% identity score which confirmed observations made with the morphological identification. Flies belonging to the stomoxyine can be a source of problems in feedlots and dairies where they easily breed in moist soil and substrates [35]. Extreme biting activity could result in reduced weight gain and milk production.

In the current study, LSDV and *A.marginale* were detected by PCR on whole fly homogenates. This finding is a demonstration of the possible role of *S. calcitrans* as a mechanical vector in the transmission lumpy skin disease virus and *A. marginale*. Previous studies have reported stable flies as mechanical vectors of many other pathogen-causing diseases such as trypanosomiasis, anthrax, besnoitiosis, West Nile virus disease, Rift valley fever, bovine herpes virus disease, vesicular stomatitis, bovine leukosis, as well as equine infectious anaemia disease [7,3,36,37].

The overall occurrence of *A. marginale* DNA in this study was 22.6% across the three study locations. The detection of *A. marginale* in this study, a known tick-borne pathogen from *S. calcitrans* at the sampled locations confirms the possibility of these flies acting as mechanical vectors for the pathogen. Recently, a prevalence of 29.1% was observed in stable flies collected from a tick free herd in Mexico [38]. According to Alonso et al. [39], the geographical distribution of bovine anaplasmosis is not exclusively determined by its tick vector as observed in some regions of Latin America where the disease is more widespread than the biological vector, *Rhipicephalus* (*Boophilus*) spp. This indicates the existence of other vectors or different modes of transmission, particularly mechanical transmission by biting flies [39]. To further confirm our observation of probable mechanical transmission, it was previously demonstrated via experimental studies that mechanical transmission of *A. marginale* by *S. calcitrans* was possible [7]. Furthermore, a correlation between antibodies to *A. marginale* in dairy herd and presence of stable flies was observed in Costa Rica [6]. Additionally, a recent outbreak of bovine anaplasmosis in dairy cattle in Brazil was associated with the presence of large number of *S. calcitrans* [40]. Results in the present study suggest that *S. calcitrans* are harbouring *A. marginale* and are possibly transmitting the disease in bovines from the sampled feedlots.

LSD is endemic in Sub Saharan Africa and parts of North Africa and Middle East [3,20]. Blood sucking arthropod vectors such *S. calcitrans* (stable flies), tabanids (horse flies), ticks and mosquitoes have been implicated as mechanical transmitters of LSDV [20]. Our study detected 15% presence of LSDV DNA from *S. calcitrans* collected from a feedlot in Potchefstroom. A

significant correlation was observed between LSDV outbreak and *S. calcitrans* abundance where the *S. calcitrans* population peaked in the months of the lumpy skin disease onset in Israeli dairy farms [41].

*Rickettsia* spp. are regarded as emerging tick-borne pathogens, but so far data on the prevalence rates in biting flies particularly *S. calcitrans* are rare. Although *Rickettsia* spp. were not detectable by PCR in the current study, it was detectable by metagenomics analysis from 16S next generation sequencing [42], which may indicate that the titter of the rickettsial DNA was below the sensitivity of the PCR protocol used in this study. The potential ability of *S. calcitrans* to mechanically transmit pathogens also depends on the level of bacteraemia in the host bloodstream and the time in between interrupted feeding.

*Rickettsia* and *Ehrlichia* are tick-borne pathogens that infect animals and humans. There is ample literature available on the transmission of *Ehrlichia* and *Rickettsia* spp. by ticks. However, there is a lack of information on the role of *S. calcitrans* in the transmission of the pathogens. We attempted to detect the presence of both pathogens DNA (*Ehrlichia* spp. and *Rickettsia* spp.) using PCR. All DNA samples from all sampled locations were PCR negative. Previously, it was demonstrated that *S. calcitrans* are able to ingest and retain viable *Ehrlichia risticii* although they are unable to transmit the pathogen [43]. According to Levine et al. [44], biting flies have been well documented in the transmission of pathogens, but no *Ehrlichia* spp. is known or documented to be carried or transmitted by flies.

## Conclusions

This study has demonstrated the presence of LSDV and *A. marginale* DNA from *S. calcitrans* collected in 3 South African feedlots. This is the first record of *A. marginale* DNA detection from stable flies in South Africa. *Stomoxys calcitrans* in South African feedlots appear not to harbour *Rickettsia* spp. and *Ehrlichia* spp.

## Declaration of Competing Interest

The authors declare no competing interest.

## CRediT authorship contribution statement

**Nokofa B. Makhahlela:** Conceptualization, Investigation, Writing – original draft. **Danica Liebenberg:** Conceptualization, Supervision, Investigation. **Huib Van Hamburg:** Conceptualization, Writing – review & editing. **Moeti O. Taioe:** Formal analysis. **ThankGod Onyiche:** Writing – review & editing. **Tsepo Ramatla:** Formal analysis, Writing – review & editing. **Oriel M.M. Thekisoe:** Conceptualization, Supervision, Investigation, Writing – review & editing.

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