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RESEARCH ARTICLE

Epidemiological Investigation and Development of Loop Mediated Isothermal Amplification for the Diagnosis of Ovine Theileriosis

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A B S T R A C T

Small ruminant industry is threatened in tropical and sub-tropical countries due to a *Theileria* (*T.*) *lestoquardi*, a haemoprotozoan. A Loop-Mediated Isothermal Amplification (LAMP) assay was developed for diagnosis of *T. lestoquardi,* a major cause of ovine theileriosis. The primers were designed based on the clone-5 sequence of *T. lestoquardi*. Four types of geo-climatic regions were selected for sampling. Towards this end, 56% sheep were infected with *T. lestoquardi* in the study area. 9.1% were found infected by using Giemsa staining technique, whereas, 13 and 17.1% by polymerase chain reaction (PCR) and loop mediated isothermal amplification (LAMP), respectively. PCR was considered a reference test to evaluate specificity and sensitivity of LAMP. The relative specificity and sensitivity of LAMP assay was 94.4 and 78%, respectively. Findings of LAMP assay revealed that the highest prevalence was detected in Bahawalpur (21.6% 19/88*100) followed in order by Jhang (17.04% 15/88*100), Sahiwal (15.09% 14/88*100) and Faisalabad (13.63% 12/88*100). LAMP assay found most suitable test for diagnosis of *T. lestoquardi*. This is anticipated that this study will provide a base-line data for caretakers and veterinary personnel to devise an improved scheme for the effective control of ovine theileriosis.

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INTRODUCTION

Malignant ovine theileriosis caused by *Theileria* (*T.*) *lestoquardi* is transmitted by *Hyalomma anatolicum anatolicum.* The *T. lestoquardi* is mostly prevalent in Central Asia, China, India, East, Middle East and North Africa, Eastern and Southern Europe with mortality rates in sheep and goats as compared to ovine/caprine Theilerial species such as *T. ovis, T. luwewnshuni* and *T. uilenbergi*) (Magzoub *et al.*, 2021). The *T. lestoquardi* was first diagnosed by an Egyptian veterinary inspector in exported Sudanese Sheep by Mason in 1914 (El-Imam *et al.,* 2015). Afterward, it was reported in other countries like Iraq (Latif *et al.,* 1977), India (Sisodia *et al.,* 1981), Sudan (El-Ghali and El Hussein, 1995), Turkey (Sayin *et al.,* 1997), Saudia Arabia (El-Azazy *et al.,* 2001), Iran (Spitalska *et al.,* 2005), Oman (Tageldin *et al.,* 2005) and Pakistan (Durrani *et al*., 2011). Recently, a dramatic increase in the import of exotic breeds of cattle in Pakistan reciprocally increased tick's population due to their natural vulnerability to tick infestation. Consequently, not only in the cattle population but in sheep, number of ticks-borne diseases have noticeably increased (Hegab *et al.,* 2020; Abid *et al.,* 2021; Mushtaq et al., 2021).

The morphological structure of *Theileria* merozoites are rod shaped, measuring 2.0 um long 1.0 um wide in stained blood or lymph node smear under the microscope. Other shapes like oval, round and ring shaped also common. Sometimes multiple parasites per one erythrocyte can be observed. Two types of schizonts (also known as Koch's body) named macroschizonts (having 8 small nuclei) and microschizonts (36 small nuclei) measuring 8.0 µm in size are present (Mans *et al.,* 2015).

Theileria life cycle characterized by schizonts and piroplasm forms in their lymphoid tissues and red blood cells (RBCs), respectively. *Theileria* completes its pre-erythrocytic schizogonic life stage in leukocytes and merogony stage in erythrocytes. When ticks feed on infected host RBCs (Witschi *et al.,* 2013). The RBCs lysed in the tick gut lumen and piroplasm divides into the gametocytes which leads to the fertilization also known as syngamy. A zygote having spherical shape, penetrates in gut lumen epithelial cells. After this motile form, kinete is formed as a result of the meiotic division of the parasite. This kinete break out from the gut cells of the moulted tick into the next instar and attain approach to the haemocoel. It invades salivary gland cells. Where it completes other multiplication stages (sporogony to multinucleated sporont) and the formation of sporozoites occur. These sporozoites transfer gradually into the mammalian host (Jalovecka *et al.,* 2018). Among the two types of transmissions e.g. transovarial and transstadial, the first one occurs in ticks. In mammals, after the biting of infected ticks, these sporozoites invade the lymphocytes of the mammal and convert into the schizonts. These schizonts do not have a vital role in the pathogenesis, but they can be diagnosed into the liver, spleen and lymph node (Watts *et al.*, 2016).

The most frequently observable clinical manifestations of theileriosis are high fever, enlargement of the superficial lymph node, decrease rumination, lethargy condition, anemia, labored breathing, conjunctivitis, anorexia and infected animal becomes a carrier. Experiments performed in Tunisia reveal that carrier states in animals have greater cost as compared to the losses due to the tropical theileriosis. Because carrier animals continue to disseminate the infection silently (Gharbi and Darghouth, 2011). Hence in disease prone areas there is a need to use advance techniques like polymerase chain reaction (PCR) and loop mediated isothermal amplification (LAMP) for early and correct diagnosis of the haemo-parasites for treatment, therapeutic management and adaption of the best control strategies.

Due to poor management and tick-control practices, the situation has further become worst. Additionally, laboratory diagnostic facilities are extremely limited in Pakistan. Therefore, this study was designed to know epidemiological status and development of LAMP assays for *T. lestoquardi* in Punjab, Pakistan. The relative specificity and sensitivity of LAMP technique with reference to PCR were also evaluated.

MATERIALS AND METHODS

Study Area: On the basis of variation of geo-climatic conditions, four districts *i.e.*, Sahiwal, Bahawalpur, Jhang and Faisalabad were selected (Fig. 1**)**.

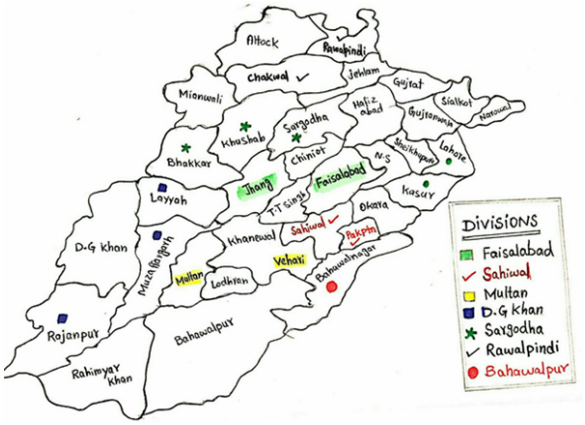

Fig. 1: A handmade map displaying location of the study areas in Punjab, Pakistan.

Sahiwal: Sahiwal Division, formerly known as Montgomery, is a city in Punjab, Pakistan. It lies on the vast Indus River plain in the densely populated region between the Sutlej and Ravi rivers. By geographical location, Sahiwal is located at 30° 40' 0" North, 73° 6' 0" East. The climate of Sahiwal district is extreme, reaching 52°C in Summer, and down to -5°C in winter. The soil of the district is very fertile. The average rainfall is about 2000 mm. In terms of human population, Sahiwal is the 21st largest city of Pakistan by population and the administrative capital of both Sahiwal District and Sahiwal Division. Livestock population ranges from large animals 1005038, small animals 521408 and Poultry 317921. Its three districts are Sahiwal District, Pakpattan District and Okara District. Sahiwal District has two tehsils; Sahiwal and Chichawatni.

Bahawalpur: Bahawalpur is located in the southeast of Punjab province. Its geographical coordinates are 29° 24' 0" North, 71° 41' 0" East. The average annual temperature is 26.1°C in Bahawalpur. The rainfall here is around 223 mm per year. In terms of human population, it is reported to be 11th largest city of Pakistan. Bahawalpur has 5 Tehsils. These tehsils are Bahawalpur, Chishtian, Ahmedpur East, Hasilpur, Khairpur, Tamewali, Yazman. The livestock counts of the city are Large animals 1327812, Small animals 1287689 and Poultry 375925.

Jhang: District Jhang is located at the convergence of Chenab (at the East) and Jhelum. By geographical location Jhang District is located at 31.27 81^0N latitude and 72.33170E longitude with average rain fall of 286mm and temperature 37°C. By human population overall in Pakistan it's considered as the 18^{th} largest city. Jhang is the word derived from Sanskrit which means Janglaor Jungle (rough or arid territory). Jhang is also rich in livestock population. It has four Tehsils e.g. Shorkot, Ahmad Pur, Athara Hazari and Jhang itself. Upon nose count, there is about 20748.497 million small ruminant's population in District Jhang. Mainly sheep are used as a source of mutton and wool production in Pakistan.

Faisalabad: Faisalabad, formerly known as Lyallpur named after the founder of the city, lies in northeast of Punjab. Its geographical location is 31° 25' 0" North, 73°

5' 0" East. The average annual rainfall is only about 375 millimeters. By human population, it is 3rd largest city of Pakistan. Faisalabad is rich in livestock population. The Livestock Population ranges from Large animals 1533586, Small animals 615,894 and poultry: 285492. Faisalabad has 5 Tehsils. These tehsils are Faisalabad, Sumandri, Tandlianwala, Jaranwala & Chak Jhumra.

Selection of Animals: Apparently healthy sheep of either sex and breed, more than 6 months old were selected. The samples were collected at the time of slaughtering in the abattoirs in EDTA containing vacutainers. Total sample size was 450, out of these, 350 (~352; 7-10 ml blood/sample; n=88 samples/district) were used to analyze the prevalence and rest of the 100 samples were used to tabulate the comparison to specificity and sensitivity of the LAMP as compared to PCR. The study was performed from April through August, 2020.

Giemsa Staining: Fresh thin blood smear was prepared on a microscope slide and fixed with pure methanol for 30 seconds. The slide has been dipped in a readily prepared 5% Giemsa stain solution for 15–20 minutes, then flushed it under the tap water and left it to dry (Coles, 1986).

Genomic DNA Extraction: Whole genomic DNA from blood samples were extracted by using a commercially available DNA extraction kit (QIAGEN). Genomic DNA has been purified according to the instructions provided by the manufacturer's company. The protocol for genomic DNA extraction was followed accordingly concerning the quantification of starting material. 200 µl of whole blood was homogenized with 20 µl proteinase K and 400 µl of lysis solution. The homogenate was incubated at 56°C for 10min and was occasionally vortexed. This is followed by addition of 200 µl of absolute ethanol. After mixing the lysate is shifted to a GeneJet genomic DNA purification column and DNA was adsorbed onto the GeneJet silica-gel membrane during four centrifugation steps. Then, the bonded DNA was washed by centrifugation using two different washing buffers. The purified DNA was eluted from the Genejet genomic DNA purification column in 200 µl of elution buffer (Amira *et al.*, 2018).

PCR Amplification: The 730 bp sequence from 18S rRNA gene of *T. lestoquardi* was amplified using a set of oligonucleotide primer [fwd 5'-GTGCCGCAAGT GAGTCA-3' and rev 5'-GGACTGATGAGAAGAC GATGAG-3'] through PCR which was performed in a final reaction volume of 25 µl (Saeed *et al*. 2015). It contained 13 µl of Dream Taq Green PCR Master Mix (2x) (Thermo Scientific; Catalog number: K1081), 2µl (10 pmol) of forward and reverse primers, 5 µl of template DNA isolated from field samples, 2.0µl of 10X PCR buffer, 0.5 µl of 10 mM dNTP mix, 1.5 µl of 25 mM MgCI2 (ThermoScientific). The volume was made up to 25 µl by adding the nuclease free water. The Purified *T. lestoquardi* DNA isolate from clinically infected sheep was served as a positive reference control, while the DNA isolated from the blood of a three-day-old lamb was served as reference negative control. The cycling conditions included an Initial denaturation at 96°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1min and then extension at 72°C for 1 min and the final extension was performed at 72°C for 7 min. The PCR products were visualized on 1.5% agarose gel and visualized using a gel documentation system (Syngene, U.K.). The results of PCR assay were compared with that of Giemsa stained blood smear examination and statistical analysis of the data obtained was carried out (Bhanot *et al.,* 2018). Gel electrophoresis for performed for the evaluation of amplification and the bands were compared with the standards.

Development and evaluation of Loop-Mediated-Isothermal Amplification method (LAMP Technique) for the detection of T. lestoquardi: Known positive *T. Lestoquardi* (Confirmed through microscopically and then with PCR) samples have been used for establishing of LAMP technique. LAMP assay has been carried out in a reaction mixture containing: Deoxy nucleotide tri phosphates (dNTP), betaine, Bst-DNA polymerase buffer, $MgSO_4$, ddH_2O and DNA template. The primers were prepared according to sequenced used by Salih *et al*. (2012); Forward outer (F3; 21bp) AGATACCAAGGAAACTGAAGG, Backward outer (B3; 24bp) TGTATCCTTAGGTTTTTCATGTTC, Forward inner primer (FIP; 49bp) CAGGAGAAAT AGGAGTTTCAGGTTC-CAAAGGATAAGAAAGAT GAAAAGG; Backward inner primer (BIP; 44bp) GTA TCGCACCAGAACCTCAAC-ACAGTTTCTTCTTTAT CCTGATC.

The procedure of the LAMP assay: Genomic DNA of *T. lestoquardi* was extracted as described above (Section 2.3). A total of 25 µl volume of the reaction mixture was prepared. Warmstart Colimetric LAMP Master mix (2x), ddH2O and DNA template. Forward primers (outer) F3, backward primer (outer) B3. Forward primer (inner) and backward primer (inner) FIP & BIP.12.5 µl LAMP reaction buffer, 0.9 µl of primers mixture (FIP and BIP at 40 pmol each and F3 and B3 at 5 pmol each targeting variable region of 18S RNA gene sequence).

Five µl of the extracted DNA isolated using DNA extraction kit method (Qiagen). The volume of 25 µl was made up with nuclease free water.

First vortex this reaction mixture for 10 minutes; to after this, the reaction mixture has been incubated at 60-65ºC for almost 30 min and be ended by increasing the temperature at 80ºC for 10 minutes (until the reaction was terminated). This incubation process has been carried out in the water bath. The reaction was optimized at 65ºC for 35 minutes with a stop reaction of 5min at 80ºC.

Then, the LAMP product has been subjected to Syber green dye placed on the inner side of the tube without opening to the tube. Then observe the tube colour after 10 mins. The results were analyzed through gel electrophoresis. The specificity and sensitivity also calculated in comparison with the PCR and LAMP technique. PCR was considered reliable reference test. Samples (n=100) were run to LAMP assay in comparison to PCR. The relative specificity and sensitivity of LAMP assay has been calculated by using the following formula:

$$\text{Specificity [Sp]} = \{\text{(No. of Samples negative in both tests} \div \text{Total No. of negative samples from both test)} \times 100\}$$

$$\text{Sensitivity [Se]} = \text{(No. of Samples positive in both tests} \div \text{Total No. of positive samples from both test)} \times 100$$

RESULTS

The overall prevalence of *T. lestoquardi* in Faisalabad, Bahawalpur, Sahiwal and Jhang was 43.18, 73.86, 54.54 and 56.82%, respectively. Overall prevalence noted by Giemsa staining was 9.1% (32/350), whereas PCR (Product size 730 bp) and LAMP was 13% (48/350) and 17.1% (60/350), respectively. In PCR, well number nine was positive and product size was 520 bp (Fig. 2). In LAMP assay, S1-S3 were positive (Fig. 3). Findings of LAMP assay revealed that highest prevalence was detected in Bahawalpur (21.6% 19/88*100) followed in order by Jhang (17.04% 15/88*100), Sahiwal (15.09% 14/88*100) and Faisalabad (13.63% 12/88*100). Prevalence found by Giemsa staining was 6.81, 13.63, 11.36 and 10.22%, whereas PCR 10.22, 18.18, 12.5 and 13.63% in Faisalabad, Bahawalpur, Sahiwal and Jhang, respectively. The relative specificity and sensitivity of LAMP assay was 94.4% (22/28x100) and 78% (68/72x100), respectively (Table 1).

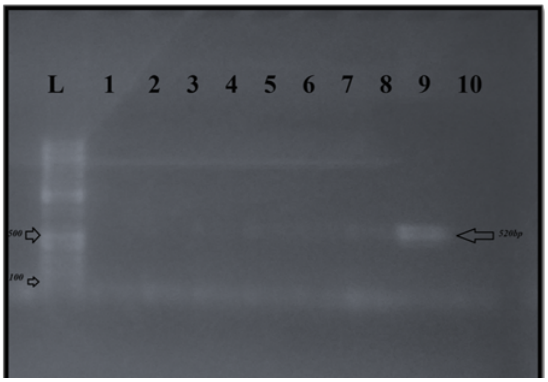

Fig. 2: PCR product for the detection of *Theileria lestoquardi*. L; 100 bp ladder. 9th well showed positive sample. Well number 1, 2, 3, 4, 5, 6, 7, 8 and 10 are the negative samples.

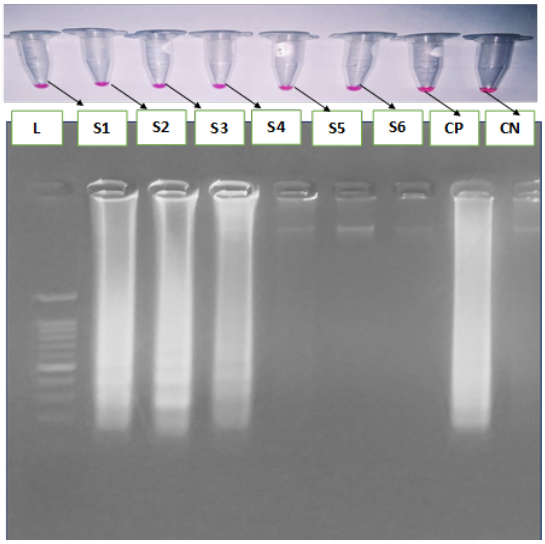

Fig. 3: LAMP product using genomic DNA obtained from *Theileria lestoquardi*. L; 100 bp ladder. S1, S2 & S3 showed positive sample. S4, S5 & S6 are the negative samples. CP showed the positive control and CN is the negative control sample.

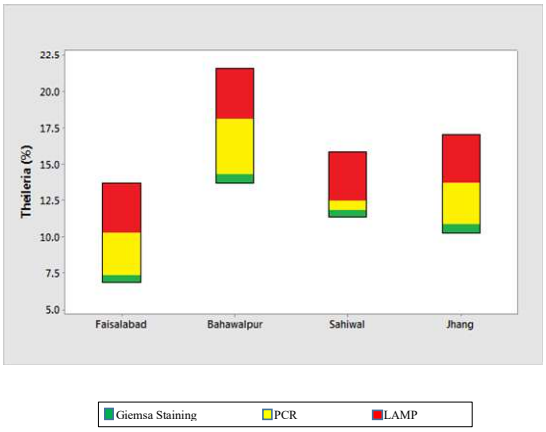

Fig. 4: Comparison of estimated prevalence of *Theileria lestoquardi* detected by Giemsa Staining, PCR and LAMP in Faisalabad, Bahawalpur, Sahiwal and Jhang (Punjab-Pakistan) in sheep and goats.

DISCUSSION

Ovine theileriosis caused by the *T. lestoquardi* is the most important tick-borne disease of ovine also called malignant ovine theileriosis which affects both sheep and goats. This disease constitutes an obstacle in the small ruminant industry in many developing countries like Pakistan (Zaman *et al.,* 2020; Khan *et al.,* 2022). The main goal of this study was to develop an time-and-cost effective diagnostic technique for the malignant ovine theileriosis technique. This diagnosis has been made on the basis of specific sequence of antigen protein named as clone-5 of *Theileria spp.* (*T. lestoquardi*). This specific protein is recently delineated and named as immunogenic protein which is recognized on the specific region of Theileria stage called schizonts. This clone-5 protein also used in the serological tests like ELISA for the diagnosis of *T. lestoquardi* (Bakheit *et al.,* 2006).

In the resource-limited countries, due to having cheap and inexpensive, Giemsa staining for the detection of blood-protozoan is used very frequently (Ahmed *et al.,* 2021; Mousa *et al.,* 2021). However, as per our studies, Giemsa staining can detect *T. lestoquardi* very poorly (9%). Low detection ability of Theileria spp. by Giemsa staining was also recorded by Soliman *et al.* (2021) and Maiti (2021). In a previous study in Pakistan, Giemsa staining as compared to PCR technique in detection of the subclinical cases *T. ovis* and *T. lestoquardi* from Multan (Punjab-Pakistan) was reported (Riaz *et al.,* 2019). Out of 463 samples, 74 samples were positive by using PCR and 25 were positive by Giemsa staining. Mohmad *et al.* (2021) found 10.85% and 82.94% prevalence by PCR and Giemsa staining, respectively. Interestingly, Islam *et al.* (2021) recorded 8.50% prevalence by PCR and could not find any positive sample by Giemsa Staining. However, clinical cases of Theileria spp. with easily detectable by Giemsa staining (Gökpinar *et al.,* 2021). Differential

Table 1: Cross-tabulation between sensitivity and specificity of LAMP and PCR technique

		PCR		
		Positive	Negative	Total
LAMP	Positive	22	6	28
	Negative	4	68	72
	Total	26	74	100

Results of relative specificity of LAMP counted by using the formula mentioned above 68/72*100= 94.4%. Results of related sensitivity of LAMP counted by using the formula mentioned above 22/28*100= 78%.

diagnosis at the species level could not be achieved in this study by Giemsa staining. Many researchers could not differentiate at species level by just using Giemsa staining technique (Agrawal *et al.,* 2021; Mirahmadi *et al.,* 2021).

This study confirms the higher sensitivity and specificity of LAMP to detect *T. lestoquardi* at isothermal conditions in one-hour time duration than PCR. For the assessment of sensitivity of LAMP against PCR (Reference test), 100 DNA samples were utilized. Twenty-eight samples were positive by LAMP assay and 26 samples were tested positive by PCR technique. Whereas 6 samples tested positive by LAMP assay were negative by PCR and four samples tested positive by PCR were tested negative by LAMP assay, however, 68 samples were tested negative by both techniques. Above comparison showed the relative specificity and sensitivity of LAMP assay as 94.4 and 78%; respectively. These results are in agreement with Hassan *et al.* (2019). They found 96.4% specificity and 68.1% sensitivity of LAMP as compared to PCR. Previously, Salih *et al.* (2011) reported 92.1% relative sensitivity and 87.5% specificity of LAMP as compare to PCR. Wang *et al.* (2010) also found LAMP (45.4% prevalence) more efficient than PCR (41.5% prevalence).

In these three diagnostic techniques, Giemsa staining, PCR and LAMP, the prevalence was observed highest in the same order i.e. Bahawalpur then Jhang, Sahiwal and least in Faisalabad. The difference in the prevalence rate might be associated with various management and housing system of the animals, in addition to geo-climatic conditions. The education level of the farmers also varies in these regions. Being located at far from the federal and provisional centers, Bahawalpur and Jhang are under-privileged as far as education and other diagnostic facilities are concerned. Education level is 48, 46, 61, and 69% in Bahawalpur, Jhang, Sahiwal and Faisalabad (https://punjab.gov.pk/district_profile). During this study, less awareness of acaricides and tick repellents was noticed in the same order as rate of prevalence described above. The regions with similar geo-climatic conditions as Bahawalpur (Abid *et al.,* 2021; Parveen *et al.,* 2021) already reported for higher load of Theileria spp. than other parts of Pakistan (Mohsin *et al.,* 2021; Ullah *et al.,* 2021). The false-negative result may be discouraged to the farmers for creating lesser inclination. Timely diagnosis with fewer chances of false negative.

Conclusion: First epidemiological study was carried out in Pakistan on ovine theileriosis based on LAMP results. Giemsa staining technique was found least reliable for diagnosis of *T. lestoquardi* in random sampling. PCR and LAMP are efficient for screening purposes. PCR is a relatively expensive, time consuming and highly complicated procedure for diagnosis of *T. lestoquardi.* LAMP technique being time-and-cost efficient and easy to use, may be played a significant role in the timely reporting of *T. lestoquardi*. Use of Giemsa staining technique for screening of ovine theileriosis may be replaced by PCR and LAMP.

Authors contribution: MAZ and FAA conceived the idea, designed the project and supervised the work execution. AR, AA, KH, MAR, SA, FS, RMM and MO involved in data analysis, interpretation and write up of the manuscript. UM executed the work and synthesized the manuscript, SM and FAA helped in getting the work done. All authors approved the manuscript.

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