Occurrence of Total Aflatoxin and Zearalenone in Dairy Cattle Concentrate Feeds in Malawi

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Abstract

Background The study was conducted to determine occurrence and levels of total aflatoxin and zearalenone in concentrate feedstuffs for dairy animals from selected farms in Malawi. Methods A total of 130 concentrate feedstuff samples were collected in paper bags from 113 randomly selected farms in the three agroecological zones (representing high, mid and low (lakeshore) altitudes) from October and December 2019. Each feed sample was ground and analyzed for total aflatoxin and zearalenone using VICAM Fluorometer Method procedures. Results About 85% of samples comprised of corn (maize) bran (CB), 8% for pigeon pea (PP), 2% for dairy mash, 1% for soybean and <1% for sunflower, rice bran (RB) and rice bran mixed with maize bran respectively. 75% of corn bran and 100% of dairy mash and rice bran samples were positive for zearalenone (i.e. above 100 µg/kg) while other concentrates tested negative. Incidences of total aflatoxin were 32%, 67% and 9% in CB, DM and PP bran samples respectively and negligible in other concentrates. Overall, 32% and 23% of feedstuffs had total aflatoxin concentrations above regulatory limits set by Malawi's neighboring countries (Tanzania and Mozambique) and US respectively whereas only 6% of all the concentrate feedstuffs had zearalenone concentrations above the regulatory limit enforced by EU. Levels of total aflatoxin and zearalenone in CB were not affected by agroecological zones (P=0.17 and P=0.87) respectively. Mean total aflatoxin concentration was 35.4, 28.1 and 38.2 µg/kg in High, Lakeshore and Mid agroecologies respectively whereas zearalenone concentration was 249.7, 226.8 and 243.9 µg/kg respectively. Conclusion To the best of our knowledge, this is the first study to report prevalence of various mycotoxins in dairy concentrate feedstuffs in Malawi. Aflatoxin and zearalenone contamination in dairy concentrates exist but mostly in levels within tolerable limits at least from the limited timeframe of this study. However, presence of total aflatoxin above regulatory limits in 26 - 34% of corn bran, a major concentrate feedstuff, points to the need for deliberate efforts to ensure levels are kept low in dairy animal feeds for both safety of dairy animals and consumers in Malawi.

Introduction

Contamination of livestock feeds with mycotoxins remains a global challenge. Several studies have reported presence of one or more mycotoxins in feeds and feedstuffs meant for livestock in all regions of world (Driehuis et al., 1992; Schmidts et al., 2014; Ji et al., 2014; Gutleb et al., 2015). Mycotoxins are a group of chemically diverse compounds originating from secondary metabolism in fungal molds (Fink-Gremmels, 1999; Njobeh et al., 2012). Aflatoxin and zearalenone (ZEN) that are mainly produced by *Aspergillus* and *Fusarium* species of fungi respectively are among mycotoxins of great concern in livestock production (Hussein and Brasel, 2001; Whitlow and Hagler, 2016). Among other effect, zearalenone is associated with reduced reproductive efficiencies in livestock (Weaver et al., 1986a; Weaver et al., 1986b; Kordic et al., 1992) whereas aflatoxin B_1 and its metabolite aflatoxin milk 1 (M1) are mutagenic and carcinogenic to both animals and humans (Wogan et al., 1974; IARC, 1993; Boudra et al., 2007).

Prevalence of mycotoxins is known to be highly enhanced by environmental factors such as warm temperatures and moist conditions that promote growth of toxigenic fungi (Lacey, 1991; Magan et al., 2003; Ribeiro et al., 2006; Murphy et al., 2006). These conditions would typically be expected to exist in small scale dairy production and in the feed storage facilities. Earlier studies have reported greater presence of various mycotoxins including aflatoxins, zearalenone and fumonisins in cereal and legumes for human consumption in Malawi (Matumba et al., 2009; Matumba et al., 2011; Monyo et al., 2012; Matumba et al., 2015; Mwalwayo and Thole, 2016). The dependence of dairy animals on crop-based supplemental products such as corn (maize) bran, rice bran, soybean, peanuts, pigeon pea apart from dairy mash would likely increase the intake and exposure to mycotoxins hence increased consumer risk. However, in Malawi, the occurrence of mycotoxins and the extent of this problem is not well understood at least from the small scale dairy subsector standpoint and particularly on feeds. Absence of knowledge on mycotoxins prevalence and the extent of contamination of livestock feeds puts the health of both animals and humans at a higher risk of exposure to intolerable levels of mycotoxins with potential for suppressing immune function, decreasing the response to resist infectious diseases and reducing animal performance and efficacy of vaccines and drugs (Jiang et al., 2005; Oswald et al., 2005; Berek et al., 2001; Sharma et al., 1993). Therefore, the objective of this study was to determine incidences and levels of aflatoxins and zearalenone in concentrate feedstuffs among small scale dairy farms located in three agroecological zones of Malawi.

Materials And Methods

Study area and sample size

The study was carried out in Malawi between October and December 2018. Within Malawi, the study focused on three agro-ecological zones (AEZ) namely Highlands (< 1,300 m above sea level), Mid-elevation (760–1,300 m above sea level), and Lakeshore, Middle and Upper Shire (200–760 meters above sea level) where dairy farming is predominantly practiced. In order to obtain a representative sample of the population, a multi-stage sampling technique was used to obtain a total of 113 dairy farms from which various concentrate feedstuff samples were collected in these three AEZs as follows; five milk-bulking groups were randomly selected within each agroecological zone. Milk bulking groups are associations or cooperatives comprising of up to 100 members. They are established to promote collective milk marketing and ease accessibility to various extension services including dairy management trainings (IFS, 2013). Approximately 8–11 farms per milk bulking group were randomly selected from the provided list of dairy farms. An exception was with Lakeshore AEZ where all the 20 participating dairy farms were selected from one milk-bulking group because it was the only available group at the time of the study. Therefore, 20 farms were drawn from lakeshore, middle and upper shire (Lakeshore) agroecological zone while 51 and 42 farms were included in the study from Highlands and Mid elevation agroecological zones respectively making a total of 113 dairy farms. From these dairy farms, a total of 130 concentrate feedstuff samples were collected in paper bags. For each feedstuff, multiple samples were collected and then mixed to make one sample weighing 0.5–1 kg for preparation and analysis as described in the following sections.

Mycotoxin extraction and analysis

Every feed sample was ground to pass through a 1 mm screen to provide fine and homogeneous samples for mycotoxin analysis. The ground samples were stored in plastic containers in a cool, dry place until analyzed. Total aflatoxin and zearalenone were extracted and determined using VICAM Fluorometer Method (VICAM, 2014), according to the analytical procedures detailed below.

a) Aflatoxin extraction and analysis

A sub sample of ground feed (50 g) was mixed with 10 g NaCl and place in blender jar. Then, 200 mL methanol and water at the ratio of 80:20 was added into a blender jar. The mixture was blended at for 1 minute and thereafter the extract was filtered through fluted filter paper. The filtrate was collected in a clean vessel from which 10 mL was drawn using a pipette and placed into another clean vessel. The extract was diluted with 20 mL purified water and then filtered through 1.5 μm glass microfibre filter into a clean vessel. A total of 2 mL of filtered extract was added (1 mL at a time) to the AflaTest column headspace and passed through the column at a rate of about 1 drop/second until air came through the column. The column was washed with 1 mL of purified/distilled water at a rate of 1–2 drops/second. And this step was repeated until air comes through column. High Performance Liquid Chromatography (HPLC) grade methanol (1 mL) was passed through the column at a rate of 1 drop/second in order to elute the toxin and the sample elute was collected in a glass cuvette (VICAM part # 34000). AflaTest Developer solution (1 mL) was added to the sample elutes in the cuvette and mixed thoroughly. The cuvette was placed in a calibrated VICAM Series 4EX fluorometer and aflatoxin concentration was determined after 60 seconds. The detection range of the assay was 0–50 μg/kg (VICAM, 2014). The sample elutes containing concentration of aflatoxin greater than 50 μg/kg were diluted ten folds with (1:1) volume ratio of HPLC grade methanol and AflaTest Developer solution and retested.

b) Zearalenone extraction and analysis

A sub sample weighing 20 g was mixed with 2 g of salt and placed in a blender jar. Then, 50 mL of methanol:water (80:20) was added to the mixture and blended at high speed for 2 minutes. The mixture was filtered through fluted filter paper and collected in a clean vessel. The extract was diluted with 40 mL 1 × 0.1% Tween PBS Buffer and filtered through 1 μm glass microfiber filter. Diluted and filtered extract (10 mL) was passed through ZearalaTest column at a speed of 1–2 drops per second. The column was washed by letting 10 mL 1 × 0.1% Tween PBS Buffer pass through at the speed of 1–2 drops per second. Thereafter, the column was washed by 10 mL of distilled water at a speed of 1–2 drops per second. ZEN was eluted by passing 1.0 mL HPLC grade methanol through the column at a speed of 1–2 drops per second and the elutes was collected in a glass cuvette. ZearalaTest Developer (1.0 mL) was added to elute in a cuvette and mixed thoroughly. The cuvette was placed in a calibrated VICAM Series 4EX fluorometer and readings were taken after 5 minutes. The detection range of the assay was 0–2000 μg/kg (VICAM, 2014). Sample elutes containing concentration of zearalenone greater than

2000 μg/kg were diluted ten folds with (1:1) volume ratio of HPLC grade methanol and ZearalaTest Developer solution and retested.

Statistics

The percentage incidences of total aflatoxin and zearalenone, minimum, maximum and median values for each type of concentrate feedstuff were calculated using Microsoft Excel 2010. Comparison of total aflatoxin and zearalenone levels across agroecological zones was only performed on corn bran, as it was the widely available concentrate feedstuff in dairy farms across all three agroecological zones. The total flatoxin and zearalenone data in corn bran samples were not normally distributed and were log-transformed for statistical analysis (log Aflatoxin + 1, log Zearalenone + 1) and analyzed using SAS version 9.2 (SAS Institute Inc, Cary, NC). Means were separated using Tukey's HSD. The reported means of aflatoxin and zearalenone data in corn bran samples across agroecological zones are back transformed concentrations. The level of confidence required for significance was set at $p \leq 0.05$.

Results

Incidence and concentration of aflatoxin and zearalenone in feedstuff samples

Incidence and concentration of total aflatoxin and zearalenone in concentrate feedstuffs are given in table 1. The majority of collected concentrate feedstuff samples was corn bran. From a total of 130 concentrate feedstuff samples collected, 111 (85%) were for corn bran, 11 (8%) for Pigeon pea bran, 3 (2%) for dairy mash, 2 (1%) for soybean and <1% each for sunflower, rice and mixture of corn bran and rice bran. Zearalenone levels above the limit of detection (100 μg/kg) was present in 75% of corn bran feedstuff samples, 9% of pigeon peas bran samples, 100% of dairy mash and rice bran samples but not detected in soybean, sunflower and a mixture of corn bran and rice bran. The concentration of zearalenone in positive samples ranged from 100 – 2400 μg/kg with a median of 240 μg/kg in corn bran, 130 - 410 μg/kg with the median of 160 μg/kg in dairy mash and at a concentration of 280 μg/kg in one and only positive pigeon pea bran feedstuff sample. Total aflatoxin was detected above limit of detection of 10 μg/kg in 36%, 67% and 9% of corn bran, dairy mash and pigeon pea bran feedstuffs samples respectively and not detected in soybean, sunflower, rice bran and a mixture of corn bran and rice bran. Levels of total aflatoxin in positive feedstuff samples of corn bran ranged from 10 – 410 μg/kg with a median of 37 μg/kg and 11 – 110 μg/kg with a median of 60.5 μg/kg in dairy mash. Total aflatoxin was only detected in 1 out of 9 pigeon pea bran samples at concentration of 16 μg/kg.

Table 1 Incidence and concentration of total aflatoxin and zearalenone in concentrate feedstuffs commonly fed to dairy animals in Malawi

Feed class	Sample (n)	Total aflatoxin				Zearalenone			
			Concentration (μg/kg)[2]				Concentration (μg/kg)[2]		
		Positive samples[1] (%)	Min	Max	Median	Positive samples[1] (%)	Min	Max	Median
Corn bran	111	40 (36)	10	410	37	83 (75)	100	2400	240
Pigeon pea bran	11	1 (9)	-	16	-	1 (9)	-	280	-
Soy meal	2	0 (0)	-	-	-	0 (0)	-	-	-
Sunflower	1	0 (0)	-	-	-	0 (0)	-	-	-
Dairy Mash	3	2 (67)	11	110	60.5	3 (100)	130	410	160
Corn bran+Rice bran	1	0 (0)	-	-	-	0 (0)	-	-	-
Rice bran	1	0 (0)	-	-	-	1 (100)	-	210	-

[1]Number and percentage (value in parentheses) of concentrate feedstuff samples with aflatoxin and or zearalenone concentration greater than the limit of detection (LOD).

[2]Concentration total aflatoxin and zearalenone in μg/kg of positive feedstuff samples.

Feedstuff samples with aflatoxin and zearalenone above regulatory limits

Number and proportions of concentrate feedstuff samples with either total aflatoxin or zearalenone concentration above various regulatory limits are presented in table 2. Overall, 32% of concentrate feedstuff samples had total aflatoxin concentrations above regulatory limit of 10 μg/kg set by Malawi's neighboring countries Tanzania and Mozambique, 23% did not comply with total aflatoxin regulatory limit of 20 μg/kg in dairy feeds set by US and only 6% of all the concentrate feedstuff samples had zearalenone concentrations above 500 μg/kg, the regulatory limit enforced by EU. Thirty four percent (34%) and 26% of corn bran samples had total aflatoxin concentration levels above regulatory limits set by Tanzania plus Mozambique and US respectively whereas 7% had concentrations levels of zearalenone above EU regulatory limit. Sixty seven percent (67%) of dairy mash samples (n=3) had total aflatoxin concentration levels above regulatory limits of 10 μg/kg enforced by Tanzania and Mozambique and only one sample had levels of total aflatoxin above 20 μg/kg limit followed by US.

Table 2 Proportion of positive concentrate feedstuff samples with total aflatoxin and zearalenone (ZEN) levels above set regulatory limits

Feed class	Total samples	Samples with TAF above 10 μg/kg (%)[1]	Samples with TAF above 20 μg/kg (%)[2]	Samples with ZEN above 500 μg/kg (%)[3]
Corn bran	111	38 (34)	29 (26)	8 (7)
Pigeon pea bran	11	1 (10)	0	0
Soybean meal	2	0	0	0
Dairy mash	3	2 (67)	1 (33)	0
Sunflower	1	0	0	0
Corn bran+Rice bran	1	0	0	0
Rice bran	1	0	0	0
Overall	130	41 (32)	30 (23)	8 (6)

[1]Maximum regulatory limit for aflatoxins in animal feeds enforced by Tanzania and Mozambique (FAO, 2000).

[2]Regulatory limit for aflatoxins in feeds for dairy and immature animals enforced by US Food and Drug Administration (FDA, 2000; FAO, 2000).

[3]Maximum regulatory limit for zearalenone in cattle feed established by EU (FAO, 2000).

TAF = Total aflatoxin

Total aflatoxin and zearalenone concentration in corn bran across agroecologies

The mean concentration of total aflatoxin and zearalenone in positive corn bran feedstuff samples across the High, Mid and Lakeshore agroecological zones are presented in table 3. Levels of total aflatoxin and zearalenone were not affected by agroecological zone (*p=0.17*) and *p=0.87*) respectively. The mean total aflatoxin was 35.4, 28.1 and 38.2 µg/kg in High, Lakeshore and Mid agroecological zones whereas mean concentrations of zearalenone were 249.7, 226.8 and 243.9 µg/kg respectively.

Table 3 Concentration (µg/kg) of total aflatoxin and zearalenone in corn bran samples across High, Mid and Lakeshore agroecological zones

		Agroecological zone						
		High		Lakeshore		Mid		
Mycotoxin	Samples[1](n)	Mean[2]	95% CI	Mean[2]	95% CI	Mean[2]	95% CI	p-value
Aflatoxin	40	35.4	(17.6, 71.1)	28.1	(17.4, 44.5)	38.2	(25.0, 58.3)	0.17
Zearalenone	83	249.7	(208.0, 299.7)	226.8	(160.6, 320.3)	243.9	(192.0, 309.5)	0.87

[1]Number of concentrate feedstuff samples with total aflatoxin and or zearalenone concentration greater than the limit of detection (LOD).

[2]Back transformed mean concentration of total aflatoxin and or zearalenone in μg/kg

Discussion

Occurrence of mycotoxins was high in some dairy concentrate feedstuffs and low to non-detectable in others. To the best of our knowledge, this is the first study to report prevalence of various mycotoxins in dairy concentrate feedstuffs in Malawi. It must be noted from the onset however that corn bran (CB) was apparently the major form of concentrate commonly found in these small scale dairy farms representing 85% of all feedstuffs sampled followed by pigeon pea bran at 8% and 2% for dairy mash while the rest were at < 1–1%. With this in mind, 75% of CB feed samples, and 100% of dairy mash (DM) samples were positive for zearalenone, and only 9% for pigeon peas bran (PPB) whereas zearalenone was non detectable in soybean, sunflower and a mixture of corn bran and rice bran. Similarly, total aflatoxin was detected in 36%, 67% and 9% in CB, DM and PPB feedstuffs respectively and non detectable in the other feedstuffs. These results are consistent with those from others studies that have reported high incidence of aflatoxin and zearalenone in various feed samples (Kang'ethe and Lang'a, 2009; Schmidt et al., 2014; Kosicki et al., 2016; Gutleb et al., 2015; Schatzmayr and Streit, 2013; Anukul et al., 2013). The high incidence of zearalenone and aflatoxins contamination in corn bran and dairy mash feedstuff samples observed in this study could be attributed to availability of favorable conditions for growth of *Aspergillus* and *Fusarium* fungi. The growth of toxigenic fungi and the subsequent production of mycotoxins are reported to be enhanced by environmental factors like temperature and moisture (Lacey, 1991; Magan et al., 2003; Ribeiro et al., 2006; Murphy et al., 2006). Optimal production of aflatoxin is known to occur at temperatures between 25 and 33^{o}C (Ribeiro et al., 2006; Murphy et al., 2006; OBrian et al., 2007) while zearalenone is optimally produced at temperatures ranging from 25 to 30^{o}C (Murphy et al., 2006). Located within the tropics, these conditions are prevalent in Malawi hence growth of *Fusarium* and *Aspergillus* fungi and subsequent high incidences of aflatoxins and zearalenone contamination. The low incidence of total aflatoxin and or zearalenone in pigeon pea bran, and non-detection in soybean meal and sunflower is a notable result in this study. While the factor(s) responsible for this low incidence in these feedstuffs is not known and was not the core scope of this study, some mycotoxins are reported to be more associated with certain agricultural commodities than others. For example, soybeans are generally considered resistant to *Aspergilli* colonization and aflatoxins contamination (Njobeh et al., 2009; Gupta & Venkitasubramanian, 1975) whereas zearalenone is commonly detected in corn, wheat and corn-based feeds (Driehuis et al., 1992; Schmidts et al., 2014; Lee and Ryu, 2017). Nevertheless, considering that aflatoxins and zearalenone exert synergistic effects on livestock including cattle (Jovaišienė et al., 2016; Huang et al., 2018), the high incidences of these mycotoxins in major concentrate feedstuffs for dairy animals like CB is cause for concern among small scale dairy farmers in Malawi.

Considering a wide array of adverse health effects associated with exposure to mycotoxins, several countries have established regulatory limits. Among southern Africa countries, Tanzania and Mozambique have set regulatory limits at 10 μg/kg for aflatoxins in animal feeds (FAO, 2000). The

European Union (EU) has no regulatory limits for total aflatoxin but zearalenone in cattle feeds while United States of America (US) has established limits for total aflatoxin and none for zearalenone in animal or specifically dairy animal feeds (FAO, 2000; FDA, 2000). However, Malawi is yet to set regulatory limits for mycotoxins in animal feeds. Regulations are set to prevent exposure of either livestock or humans to levels of mycotoxins that can cause adverse health effects. This study determined prevalence of feedstuff samples with total aflatoxin or zearalenone levels above several regulatory limits. Findings of this study indicate small to moderate proportion of concentrate feedstuffs with zearalenone and total aflatoxin above various regulatory limits. Overall, 32% of all concentrate feedstuff samples had total aflatoxin concentrations above regulatory limits of 10 μg/kg set by Malawi's neighboring countries of Tanzania and Mozambique while 23% was above US set limit of 20 μg/kg for dairy feeds. Contrary, only 6% of all the concentrate feedstuffs had zearalenone concentrations above 500 μg/kg regulatory limit enforced by EU. Although Malawi has not yet established these regulatory limits for mycotoxins and specifically aflatoxin or zearalenone in animal feeds, results from this study and others done in food crops from which concentrate feeds are derived (Matumba et al., 2009; Matumba et al., 2011; Monyo et al., 2012; Matumba et al., 2015; Mwalwayo and Thole, 2016) do suggest the need for setting such policy guidelines. The finding of 26–34% of CB, 10% of PPB and 67% of DM samples with total aflatoxin concentrations above various regulatory limits (FAO, 2000) raises an alarm regarding the adverse impacts these may have on small scale dairy production and safety of dairy products for human consumption in Malawi. Since corn bran is apparently the most predominant concentrate supplement among small scale farms in Malawi, their daily consumption by animals calls for urgent follow-up studies to determine residual traces in milk and dairy products commonly found on the market and associated risk to consumers.

Concentrations of total aflatoxin and zearalenone were not affected by agroecological zones from where these feedstuffs samples were taken. The mean total aflatoxin were 35.4, 28.1 and 38.2 μg/kg in High, Lakeshore and Mid agroecological zones whereas mean concentrations of zearalenone were 249.7, 226.8 and 243.9 μg/kg respectively. These results contrast those of other studies that have reported variations in aflatoxin levels in feeds and foods across micro and macro environmental conditions (Kaaya et al., 2006; Matumba et al., 2015; Sirma et al., 2016). For example, aflatoxin contamination levels in corn samples significantly decreased from moist zone (9.73 μg/kg) to dry zone (7.72 μg/kg) and lowest in highland zone (3.92 μg/kg) in Uganda (Kaaya et al., 2006). Similarly, Sirma et al. (2016) reported significantly higher mean levels of aflatoxin in corn and millet from the humid and sub-humid agroecological zones than those from the temperate agroecological zone. In Malawi, Matumba et al. (2015) reported highest aflatoxin level and prevalence in corn samples from hottest agroecological zones while zearalenone was most prevalent in corn samples collected from cool agroecological zones. Differences in micro or macro environmental conditions may favor or prohibit growth of certain fungi compared to others and this may account for the agroecological zone variations in prevalence between aflatoxin and zearalenone. The lack of agroecological zone effect on levels of total aflatoxins and zearalenone in corn bran feedstuffs observed in this study could be attributed to inter agroecological zone trading and movement of concentrate feedstuffs by dairy farmers. For example, dairy farmers in

High agroecological zone usually buy corn bran from Lakeshore and Mid agroecological zones which are major corn producing areas in Malawi. However, this assumption has not been extensively verified. Probably a multi seasonal evaluation of total aflatoxin and zearalenone in concentrate feeds may establish an insightful presentation of the trend across agroecological zones. Regardless of the reasons for the similarities in mycotoxins concentrations, the high occurrences of aflatoxins and zearalenone in feedstuffs across agroecological zones observed in this study may compromise dairy production and also puts in question the safety of milk and dairy products to consumers. Further, this suggests that dairy animals are unduly exposed to higher levels of mycotoxin contamination that in part may affect dairy production hence need for concerted efforts to mitigate the resultant effects.

Conclusion

There were high occurrences of aflatoxin and zearalenone contamination in dairy concentrate feedstuffs commonly used in Malawi. Cereal-based concentrates (Corn bran and dairy mash) had the highest incidence of zearalenone and total aflatoxin levels among all the feedstuffs sampled compared with legumes-based concentrates (PPB, soybean and sunflower) which had low mycotoxin incidences. Although most levels were within limits, a small to moderate proportion of samples had total aflatoxin and zearalenone above various regulatory limits hence cause for concern for the safety of animals and dairy products for human consumption which also calls for urgent need for mitigation measures including setting policy guidelines. Furthermore, concentrations of total aflatoxin and zearalenone were not affected by agroecological zones which either suggests the similarities in environment conditions during the study time period or trading and movement of feedstuff across agroecological zones as possible contributory factors. Overall, these results bring to light the status of dairy animal concentrate feedstuffs regarding total aflatoxin and zearalenone contamination and raises possibilities of exposure of animal and consumers to greater and intolerable levels of mycotoxins with higher risk potential for adverse health effects.

Declarations

Availability of data

The dataset used and or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

CAN conceived the study and drafted the manuscript. JCH and MB participated in statistical analysis and helped drafting the manuscript. All authors read and approved the final manuscript.

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