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Sero-prevalence study and molecular detection of *Brucella* in pigs from sero-positive animal blood in central Ethiopia

Getachew Kinfe Demena*[1], Negassa Feyesa[2], Biniam Tadesse[2], Abde Aliy[3] and Bayeta Senbeta [3]
Animal Health Institute, Sebeta, Ethiopia
*Corresponding authors: Getachew Kinfe Demena. E-mail:getchewkin@gmail.com
E-mail: *getchewkin@gmail.com, negassa.feyissa@yahoo.com, binimanu10@gmail.com, abde.aliy@yahoo.com and didigabruma@gmail.com*

Abstract

Brucellosis cause significant reproductive loss in the swine industry worldwide however is a neglected disease in Ethiopia. It is therefore evident that researches of determining the status of the disease for intervention to combat in pig rearing areas. A cross-sectional study was conducted from April to December 2020 in central Ethiopia four areas selected purposively based on the presence of pig farms. 545 pig sera sample were collected and tested using Rose Bengal plate test (RBT) and indirect Enzyme Linked Immuno Sorbent assay (ELISA) to detect antibodies against *Brucella* infection. 21clots samples with sero positive sera and 11 blood samples from animals with previous abortion history were screened using PCR assay. Accordingly the study revealed 3.48 %(19/545) sero-prevalence of the disease but all samples screened using the IS711 target gene were found negative. Abortion was associated with *Brucella* infection with (95% CI: 0.03 0.94; P = 0.042). Lacks of awareness of the communities about Brucellosis, management problems and inappropriate handling of biological materials are significant gaps observed during the study. Further studies on isolation of the etiological agent and on zoonotic implication of the disease are advised and public awareness should be created.

Keywords: Brucellosis, pig, Prevalence, management practice, Central Ethiopia

1. Introduction

Swine brucellosis is an emerging and contagious disease caused by *B. suis* with zoonotic potential (Olsen and Tatum, 2016) and characterized by lameness and paralysis in both sexes (OIE, 2016). Due to the intercellular nature of the bacteria, brucellosis remains an endemic disease in most developing countries where livestock is a source of food and income. The World Health

Organization (WHO) classifies brucellosis as one of the world's leading 'neglected zoonosis. (Franc *et al*., 2018). Although the bacteria is host specific can infect all species of animals so wide host range resistance to the environment and it's intracellular nature makes complex epidemiology of the disease (Skendros *et al*., 2011; Yasmin and Lone, 2015). Swine brucellosis is one of the most imperative infectious causes of reproductive disease and is an economically significant cause of loss in the swine industry (Moges, 2013; Olsen and palmer, 2014). Recurrent studies have indicated occurrence of the disease with varying incidence in different species of animals except in swine in Ethiopia (Asgedom *et al*., 2016; Geresu *et al*., 2016). Therefore this work was conducted to assess current prevalence of the disease and to detect infecting *Brucella* species in pigs in central Ethiopia.

2. Materials and Methods

2.1 Study areas and study animals

The study was conducted in central Ethiopia in four different areas namely Addis Ababa, Bishoftu, Adama, and Alage (Figure1). Addis Ababa, the capital city of Ethiopia, lies at an elevation of 2300 m (7,500 ft) above sea level at 9°1′ 48″N latitude and 38°44′24″E longitude. It has a typical highland climate with temperatures ranging from 11^0C to 24^0C. The area has a long rainy season occurring from June to September with an annual rainfall of 1184 millimeters (NMIE, 2016). Bishoftu is a town located in the East Shewa Zone of the Oromia Region and has an elevation or an altitude ranging 1850 to 1920 meters above sea level found 47.9 kilometers southeast of Addis Ababa at 8o 45′ 00″ North and 380 59′ 0″ East having the rainy season from June to September. The annual rainfall and temperature were 866 millimeters and 20^0C respectively (NMSA, 2010). Adama is a city in central Oromia which forms a Special Zone at 8.54°N and 39.27°E at an elevation ranging from 1300 to 1712 meters above sea level, at about 99 km southeast of Addis Ababa. The daily temperatures range from 12^0 to 33^0C and receive an average annual rainfall ranging from about 600 to 1150 millimeters occurring in the four rainy months Jun to September (CIA, 2009; NMSA, 2010). Algae is situated at about 217 km southwest of Addis Ababa, Ethiopia at the vicinity of lakes Abijata and Shalla, located at 32 km to West of Bulbula at the geographical coordinates of 07° 34` 59`` N Latitude and 38° 25` 33`` E Longitude. The annual rainfall of the area varies from 750 to 1200 millimeters. Its annual temperature varies from 80c to 28^0C (Tedla & Degefa, 2017). As blooding pigs is not easy, to avoid crushing Pregnant and sick pigs, piglets and nursing sows were not included in the sampling.

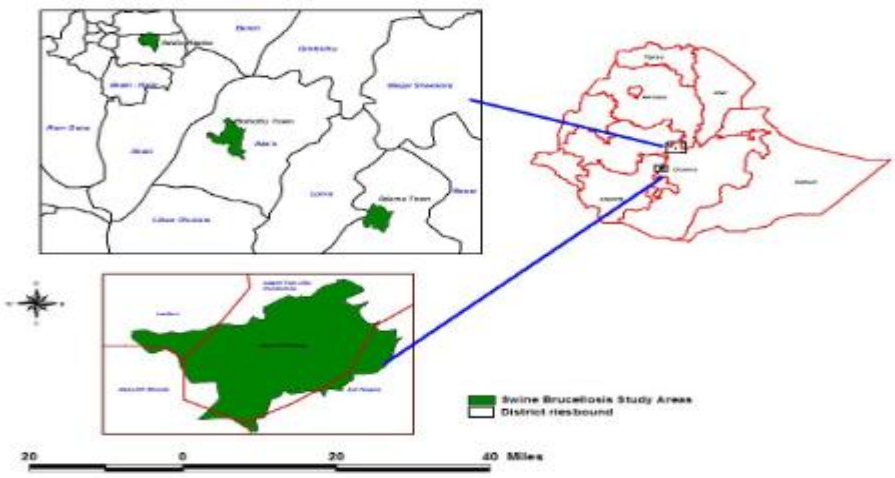

Figure **1**: Map of the study areas

2.2. Study design and methods

A cross sectional study was conducted from April to December 2020 to assess the sero prevalence of swine brucellosis in central Ethiopia. Purposive sampling technique was employed to sample each herd in the study areas. Farms were categorized based on herd size into medium composed of 10 to 50animals and large size (> 50) animals respectively to address the varying number (Geresu *et al*., 2016). To sample individual animals a simple random sampling technique was used so that every animal in the population has an equal chance of being selected in the herds. Consequently one large herd size swine farm from Alage and from Bishoftu were identified. Two medium herd size swine farms from Adama were selected. The Municipality Abattoir enterprise and one medium farm from Addis Ababa were selected.

2.3. Sample size determination

The previous sero-prevalence of the study area 4.5% (Kebeta *et al*., 2015) was considered as a base to determine sample size. The sample size was then calculated based on the formula given by (Thru-field, 007) as $N = \frac{Z^2 p(1-p)}{d^2}$

Where N = the required sample size; Z = confidence interval at 95% confidence level (Z=1.96) p= expected prevalence; d = desired absolute precision with 5% desired precision; at 95% confidence level is considered.

$N = \frac{1.96^2 * 0.045(1-0.045)}{0.05^2}$ = 66 for an area and the total sample size is 66*4=264 head of swine were supposed to be included into the study and reasonably increased by double fold to 528 to reduce standard error and to obtain better accuracy.

2.4. Questioner Survey

Questioner survey was important to assess awareness of the communities on zoonoses including brucellosis and associated risk factors so prior to blood samples collection pretested questioner was presented to respondents using local languages. Accordingly comforting their consent parallel to blood sample collection, survey data was collected from swine farm owners and concerned farm workers using one-on-one oral interviews. Management system of the farm, presence of abortion or stillbirth in pigs, disposal methods of the placenta and aborted materials, knowledge on brucellosis and other zoonoses, close contact between other animals and wildlife were collected.

2.5. Samples Collection

On farms blood samples were collected aseptically from the right side of the cranial abdominal Vein and from the ear vein of individual animals applying alcohol on the skin over the superficial veins to facilitate the identification and dilation of the vessels while at the abattoir blood were collected during slaughter from the jugular vein. About 5ml to10ml of blood samples were collected using a sterile needle and red top plain vacutainer tubes coated with clot activators. 545 animal samples from the four study areas were collected and sera were harvested into Cryo-vials without mixing with clotted blood using a micropipette. 11blood samples were collected using EDTA coated tubes for the molecular techniques from swine that have abortion history, leveled, and then transported to the diagnostic laboratory in an ice pack stored at -20^0c until processed. Format used for individual Animal sampling.

Figure **2**. Pictures during blood sample collection showing different breeds

2.6. Laboratory examinations

2.6.1. Serological tests

Rose Bengal Plate Test (RBPT) and Indirect Enzyme-Linked Immuno Sorbent Assay (I-ELISA) was carried out according to the OIE protocol (OIE, 2016). For RBT, equal volume 25 µl of sera sample and the *Brucella* antigen were mixed onto 12 wells glass slid white plate and rocked. Visible agglutination for positive samples and no agglutinations at 4 minutes as negative results was interpreted. In the I-ELISA assay, sera sample and the controls were added into 96 well polystyrene micro plate pre-coated with purified smooth *Brucella* LPS. After about 45minutes incubation paired removing the unbounded materials through the washing procedure, a multi-species horseradish peroxidase (HRP) conjugate was added to the micro wells. Finally a substrate solution (TMB) was added consequently after stopping the reaction was examined for the intensity of reaction with an automated ELISA reader at 450 nm. The signal output of blue coloration is directly correlated with the amount of antibodies in the sample.

2.6.2. Real-time Polymerase Chain Reaction (qPCR)

The Master Mix and sample preparation was done in the BSL2 safety cabinet. Blood samples collected previously from pigs with abortion history and *Brucella* sero-positive clots were cut into small pieces using tissue homogenizer then mixed separately into 20µlof proteinase K and then 80µl and 200µl of lysis buffer (AL) was added to the clot and blood samples respectively into the bottom of a 1.5ml micro-centrifuge tube. To yield a homogeneous solution and ensure efficient lysis contents were mixed thoroughly by Vortexing for 15sec and subjected to digestion decontamination washing and elution steps as per the manufacturer's guiding standard The PCR reaction was done in 20µL of PCR mix + 5µL of DNA template (samples) = 25µL reaction into 96-well PCR plates using Taq-man polymerase for IS711 Oligo-nucleotide primer sequence of forward and reverse primers sets given in Table1 (Saddique *et al*., 2019). The amplification of the genomic DNA and Real-time fluorescence detection was carried out on a Step One Plus™ Real-time PCR System (Applied Bio systems®, Germany). Data scores were determined by visual inspection of graphical curves and cycle threshold (CT) values.

Table 1 Primer & probe sequences used in real time PCR assay for detection of *Brucella*

Gene Target	Genus (Species)	Primer Sequence	Oligo
IS711	*Brucella*	5´-GCT CGG TTG CCA ATA TCA ATG C-3´	Forward
		5´ GGG TAA AGC GTC GCC AGA AG 3´	Reverse
		FAM-AAGCCA ACA CCCG GCC ATT ATGGT1 BHQ-1	Probe

2.7. Data management and analysis

Survey data and those obtained from laboratory investigations were recorded and coded using a Microsoft Excel spreadsheet (Microsoft Corporation) imported to the Statistical Package for STATA Version 13.0(Stata Corp, College Station, Texas) for conducting suitable statistical analyses. Brucella Sero-prevalence was calculated as the number of sero-positive samples divided by the total number of samples tested. Associations of sero-positivity with the risk factors such as age, sex, breed, management system, abortion history, and agro-ecology were analyzed using univariate logistic regression where $p<0.05$ indicates significance for the risk factors predicted. Variables below 0.3% (Age, breed, and abortion history) were analyzed using multivariable logistic regression to identify the potential risk factors associated with Brucella infection. Questioner data were stated using descriptive statistics. The molecular data scores were accepted by visual inspection of graphical plots and cycle threshold (CT) values.

2.8. Ethical clearance

All procedures were carried out according to the experimental practice and standard approved by the Animal Research Scientific and Ethics Review Committee (ARSERC) at Animal Health Institute for ethical approval with Reference. No. ARSERC/EC/007/24/11/2020 to minimize unnece ssary suffering therefore during sample collection animals **was** handled carefully.

3. Results

3.1. Serological Results

3.85% (21/545) prevalence of swine brucellosis was reviled using Rose Bengal Test (RBT), and 19 animals were confirmed positive for anti-*Brucella* antibodies using Indirect Enzyme-Linked Immuno-Sorbent Assay (ELISA) giving a sero-prevalence of 3.48 %(Fig2).

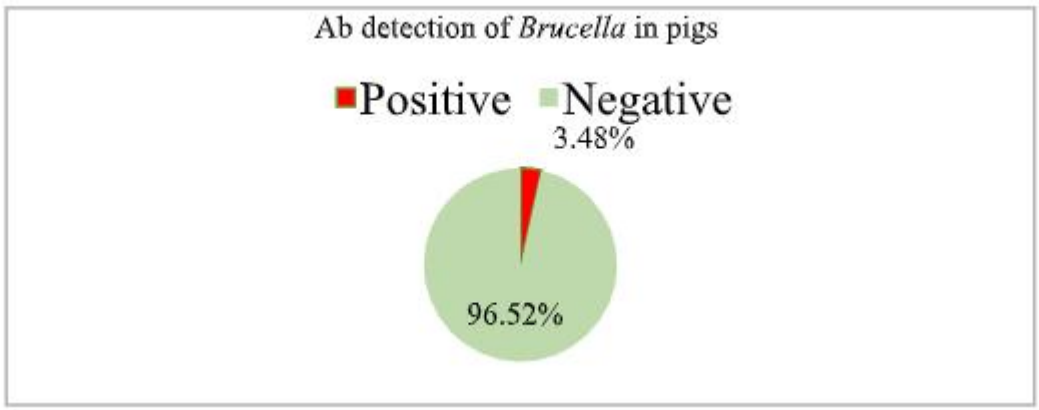

Figure **3**: Sero-prevalence of swine brucellosis in central Ethiopia

3.1.1. Host related sero prevalence using univaria te logistic regression analysis

Host related sero-prevalence of pig brucellosis was analyzed using univariate logistic regression (Table2).Some variables were statistically associated with prevalence of the disease and therefore analyzed further using multivariable logistic regression

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Table 2: Univariate logistic regression analysis of risk factors associated with pig *Brucella* Sero-positivity

Variable	Category	No. of Sample tested	Total Positive (%)	CRUDE OR (95% CI)	P value
Sex	Male	216	7(3.2%)	* 1.13 (0.44-2.92)	0.80
	Female	329	12(3.6)		
Age	Young (1year)	296	8(2.7%)	*1.66 (0.66-4.20)	0.28
	Adult (> 1year)	249	11(4.4%)		
Breed	White	344	12(3.5%)	1.02 (0.32-3.23)*	0.04
	Gray	117	4(3.4%)		
	B. spotted	84	3(3.6%)	1.05 (0.23-4.80)	0.06
Mgt System	Intensive	435	0		
	Int/Semi ints	110	19(17.3%)		
Abortion History	Aborted	11	2(18.2%)	6.76 (1.36-33.69)*	0.02
	Non Aborted	534	17(3.2%)		
A/ecology	Highland	110	19(17.3%)		
	Midland	342	0		
	Lowland	93	0		
Total		545	19(3.48%)		

**: Stands for no value. OR: odd ratio; CI: confidence interval at 95%*

3.1.2. Multivariable Logistic Regression analysis of risk factors associated with pig Brucella Sero-prevalence.

As indicated below in table6 based on the results of univariate logistic regression analysis for different risk factors p-values less than 0.3% such as age, abortion history, and breed were analyzed further using multivariable logistic regression. Consequently, the result shows that abortion history was potentially associated with sero-prevalence of Brucellosis in pigs.

Table 3: Multivariable logistic regression analysis of risk factors associated with pig *Brucella* Sero-positivity

Variable	Category	tested sample	Positive (%)	ADJUSTED OR (95% CI)	P-value
Age	Young	296	8(2.7%)	*0.7(0.27-1.9)	0.48
	Adult	249	11(4.4%)		
Breed	White	344	12(3.5%)	1.01(0.31-3.26)	0.98
	B. Spotted	84	3(3.6%)	0.89(0.19-4.19)	0.88
Abortion history	Aborted	11	2(18.2%)	0.17(0.03-0.94)	0.04
	Non-aborted	534	17(3.2%) a		

3.1.3. Farm Management and ecology factors related sero-prevalence

Univariate logistic regression analysis shows the association of risk factors with pig *Brucella*. All the sero-positive animals were from the highland agro-ecology areas with 19(17.3%). Regarding the production system the sero-positive animals were from both intensive and semi-intensive production systems.

3.2. Molecular results

Clots of blood collected from swine with abortion history and sero positive clots were screened for the detection of *Brucella* infection using target genes IS711 were found negative**.** cycle threshold value ≤ 31.42 for IS711, IPC, and for appropriate positive controls and samples in the run is considered Positive.

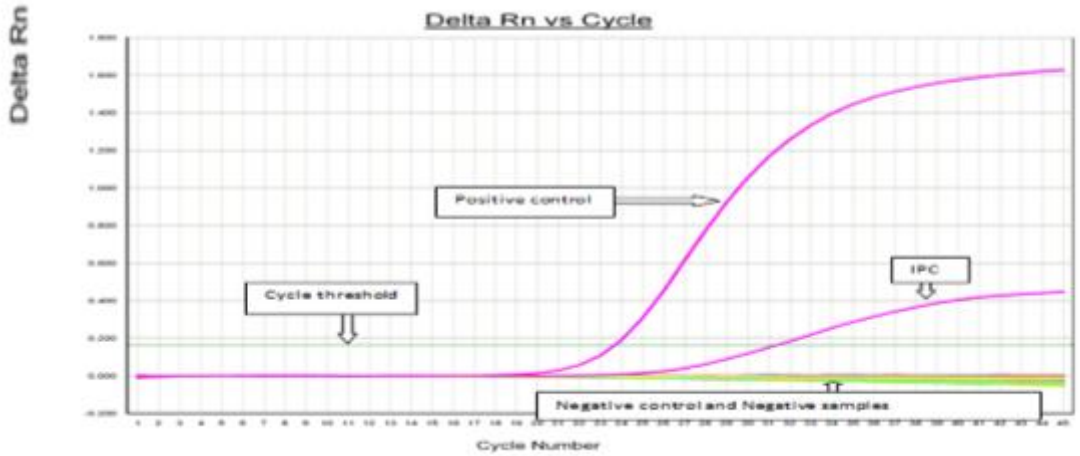

Figure 4: Real time PCR amplification patterns of controls using *Brucella* IS711

3. 3. Results of questionnaire Survey

3.3.1. Socio-demographic characteristics of the respondents

Socio-demographic characteristics of the communities gathered during the field survey shows that 52.2% of the respondents' ages between 31-45 years and the young people account for 34.8% who interviewed to assess their awareness and practices towards brucellosis. The majority of farm workers was male members of the households and was primary school level (Table 4).

Table 4: Socio-demographic characteristics of the respondents

Demographic variables	Category	Number	%
Gender	Male	15	65.2
	Female	8	34.8
Age	18- 30	8	34.8
	31-45	12	52.2
	> 45	3	13
Marital status	Marred	11	47.8
	Single	12	52.2
	College/university	5	21.7
Educational level	Secondary	6	26.1
	Primary	9	39.1
	Informal	3	13
Responsibility in farm	farm owners	4	17.4
	concerned workers	19	82.6

3.3.1. Job related risks, Knowledge and practices of respondents on health management and brucellosis

Questionnaire was provided to the owners and concerned workers in the study areas to assess occupational risks and their awareness about zoonotic diseases particularly brucellosis. Quite a few respondents had no detailed knowledge about zoonotic brucellosis, disease transmission through direct contact/ handling of fetal and aborted materials (Table 5).

Table 5: Job related risks, Knowledge & practices of respondents on health management and *Brucellosis*

Variable	Category	No	%
Knowledge on zoonotic disease	Yes	13	56.5
	No	10	43.5
Awareness about diseases transmitted during handling of fetal and aborted materials	Yes	8	34.8
	No	15	65.2
Knowledge on brucellosis	Yes	7	30.4
	No	16	69.6
Handling of fetal and aborted materials	Burning	3	13
	Deep dump	12	52.2
	Dispose to the environment	8	34.8
Assisting parturition	With protective glove	15	65.2
	Without p/glove	8	34.8

4. Discussion

The present study shows a 3.48% sero-prevalence of swine brucellosis in the study areas which is in line with 3.57% in East Shoa Oromia Regional state (Girmay (2018) but slightly lower than the previous report 4.5% (Kebeta *et al*. (2015). This may be due to the difference in testing methods that they used RBT while we used ELISA. This finding was also closely in agreement with 3.90% sero prevalence in Nepal (Sharma *et al*. (2017) using ELISA. Female animals showed a higher prevalence (3.6%) of brucellosis than males (3.2%) and a higher prevalence of the disease in adult pigs (4.4%) was observed than young (2.7%). Animals *Brucella* sero-positivity with previous abortion history was compared with non-aborted pigs resulted in (18.2%) and 3.2%) respectively with (95%CI: 1.3633.70; P = 0.02) using univariate logistic regression analysis. The Bactria can localize in the reproductive tracts as the production of sex hormones erythritol, a 4 sugar stimulates growth of the bacteria in either sex so susceptibility increases after sexual maturity and pregnancy (Ifa *et al*., 2012; Olsen and Tatum, 2016). This is why abortion was associated with sero- prevalence of the disease with (P = 0.042) using multivariable logistic regression analysis.

Many factors can affect the PCR assay such as samples of concern like blood samples often contains low amounts of nucleic acids and are the main analytical challenges together with testing assays limitations (Hedman and Rådström, 2013). In blood sample red blood cells accounts only about 45% of the total blood volume the rest 55% is composed of the white blood cells and the liquid plasma. IgG and EDTA are potential PCR inhibitors that have been identified in the blood (Alberts *et al*., 2015).

Heparin and ethanol are known PCR inhibitors th at inhibit Taq DNA polymerase and EDTA acts b y chelating Mg2+ ions (Anna L *et al.*, 2019). On the other hand, a negative result in PCR may be due to the absence of the bacteria in the blood samples (Godfroid et *al.*, 2011) because the presence of anti-*Brucella* antibodies does not necessarily mean that the animals have an active infection at the time of sampling but also suggests exposure to *Brucella* infection (Godfroid *et al.*, 2010). All farms in the study areas use a natural breeding system which is safe than artificial insemination in the dissemination of the disease but purchasing of new pigs for mating without brucellosis testing was practiced which might make suitable conditions for the spreading of the disease through the herd. The study also attempted to identify threats related to occupational risks. The young people account for 47.8%, have no experience and knowledge of zoonotic diseases and about 70% of the pig rearing participants had not heard of brucellosis and so occupation related groups have a chance of exposure to infection because swine brucellosis in humans most often is an occupationally related disease (Swai and Schoonman, 2012; Zakaria *et al*., 2018).

5. Conclusion and recommendation

Lack of surveillance, infecting wide varieties of animal species, and intercellular nature of the bacteria could make the disease endemic, and possess zoonotic consequences. Prolonged bacteremia and the ability of venereal transmission are the uniqueness of B. suis in swine considerably different from characteristics of brucellosis in mammals caused by B. abortus or B. melitensis hence statistical analysis indicates abortion history ($p < 0.05$) has a considerable effect on the overall prevalence of the disease.

Subsequently, inappropriate handling of biologica l materials causes contaminating the environment and further spreading the disease. Therefore, based on the present findings and the above conclusions, the following recommendations were forwarded.

Further studies in the prevalence and zoonotic implication of the disease

Further works in the isolation and characterization of brucellosis in swine.

Public awareness of zoonosis, safety measures, and job-related hygiene are very important.

6. Acknowledgments

The authors would like to express their gratitude to Animal Health Institute (AHI) for its support. The authors acknowledge the respective districts of animal health staff and livestock owners for their cooperation and technical support during field sample collection.

7. Ethics approval and consent to participate

The current study involved only animal samples. Research license was provided by Animal Research Scientific and Ethics Review Committee of the Animal Health Institute, Sebeta, Ethiopia. This study did not involve human participants. Written informed consent was obtained from the herd owners to take samples from their animals which were documented. Confidentiality of data obtained and the scientific morality was considered

8. Availability of data and materials

Due to the confidentiality agreements made, the datasets generated and/or analyzed during the

study are not publicly available but could be accessible from the corresponding author on reasonable request.

9. Consent for publication

Not applicable

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