सभी जानवरों के अधिकारों की रक्षा हेतु समर्पित एक राष्ट्रीय संस्था
NATIONAL ORGANISATION DEDICATED TO PROTECTING THE RIGHTS OF ALL ANIMALS

PETA

PEOPLE FOR THE ETHICAL TREATMENT OF ANIMALS

PETA India
PO Box 28260
Juhu, Mumbai 400 049
(22) 4072 7382
(22) 2636 7383 (fax)

Info@petaindia.org
PETAIndia.com

Shri Naveen Patnaik
Hon'ble Chief Minister of Odisha

17 June 2020

Via e-mail: cmo@nic.in

Subject: Request to prohibit the use of elephants to pull chariots for Rath Yatra and in all other religious processions

Dear Shri Patnaik ji,

I'm writing from People for the Ethical Treatment of Animals (PETA) India on behalf of our more than 1.5 million members and supporters to request that you prohibit the use of elephants to pull chariots for Rath Yatra (Gundicha Yatra of Lord Shree Jagannath at Puri), should the festival go ahead on 23 June. We also urge you to **end the use of elephants in all religious processions in the future** on account of animal welfare and public health concerns.

Although the Honourable Orissa High Court observed in its 9 June 2020 judgment passed in Writ Petition (Civil) No 12494 of 2020 (**Annexure 1**) that "deploying heavy duty machineries or any other means like elephants, than the men power, for pulling the Chariots/Rath, would obviously obviate the necessity of involving large number of persons, which number could be in many hundreds", this was merely an observation, not a direction from the court. In fact, the High Court specified that "this aspect should be duly considered by the State Government while taking a decision for holding Rath Yatra, *consistent with the guidelines issued by the Central Government and the State Government*" [*emphasis added*]. The use of elephants to pull chariots in religious processions causes them unnecessary pain and suffering, as they are forced walk and stand for long periods in the heat and they find the loud environment distressing. This is a direct violation of The Prevention of Cruelty to Animals Act, 1960, and the 2008 central government Guidelines for Care and Management of Captive Elephants (**Annexure 2**).

Such use of elephants for processions is also contrary to recommendations made in a research paper (**Annexure 3**) published by the Centre for Cellular & Molecular Biology – a premier research organisation under the aegis of the central Ministry of Science and Technology. The paper indicates that elephants experience extreme stress when they are made to participate in long, tiring religious processions, which can lead to infertility, hyperglycaemia, suppression of immune responses, imperfect wound healing, and neuronal cell death. It also notes that forcing them to carry heavy loads – as is the case in most processions – can multiply their concentration of stress hormones by as much as three times the basal levels and that elephants kept in captivity at temples or zoos have poorer health than those living in their natural habitat.

Furthermore, the use of elephants for any religious processions should be avoided altogether, as it may result in the transmission of tuberculosis (TB) to humans. A 2018 Animal Welfare Board of India evaluation report on captive elephants used for rides in Jaipur states that 10% of them were found to be reactive for TB. A scientific study of 600 elephants in southern India indicates a high prevalence of asymptomatic *M*

Affiliates:
• PETA Asia
• PETA Australia
• PETA Foundation (UK)
• PETA France
• PETA Germany
• PETA Netherlands
• PETA US

Registered Office:
F-110, 1st Floor, Jagdamba Tower
Plot No 13, Community Centre
Preet Vihar, New Delhi
110 092

CIN: U74899DL2000NPL103217

PEOPLE FOR THE ETHICAL TREATMENT OF ANIMALS

PETA India
PO Box 28260
Juhu, Mumbai 400 049
(22) 4072 7382
(22) 2636 7383 (fax)

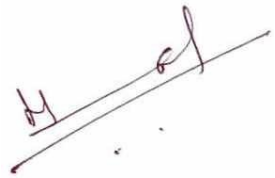
Info@petaindia.org
PETAIndia.com

tuberculosis infection. Another study found human-to-elephant and elephant-to-human transmission of *M tuberculosis* between mahouts and captive elephants. A study of 800 elephants and their mahouts over three years established cross-species transmission. In addition, a recent paper confirmed cases of TB in three wild elephants in southern India, caused by a spillover from humans (i.e. reverse zoonosis). (These studies are appended as **Annexure 4**.)

In light of the reasons stated above, we request that you prohibit the use of elephants to pull chariots in Rath Yatra and in all other religious processions in the future.

Thank you for your time and your consideration of this important matter. May I please hear from you? I can be reached at ManilalV@petaindia.org or on +91 9910817382.

Sincerely yours,



Dr Manilal Valliyate, CEO

cc: csori@nic.in; forestenv2016@gmail.com; bkarukha1962@gmail.com

Affiliates:
• PETA Asia
• PETA Australia
• PETA Foundation (UK)
• PETA France
• PETA Germany
• PETA Netherlands
• PETA US

Registered Office:
F-110, 1st Floor, Jagdamba Tower
Plot No 13, Community Centre
Preet Vihar, New Delhi
110 092

CIN: U74899DL2000NPL103217

AFR

IN THE HIGH COURT OF JUDICATURE FOR ORISSA AT CUTTACK

Writ Petition (Civil) No.12494 of 2020

Surendra Panigrahi ... Petitioner

-Versus-

State of Odisha & Others ... Opposite Parties

Advocate(s) who appeared in this case by Video Conferencing mode:-

For Petitioner	:	M/s. S.K. Padhi, N.C. Rout, & Sk. Kalimuddin, Advocates.
For Intervenor(s)	:	Dr. A. K. Mohapatra, Sr. Advocate with A.K. Mohapatra, B. Panda, S. Sarangi, A. Pati & J.S. Samal, advocates (in I.A. No.6129 of 2020)
	:	M/s. B. K. Ragada, L.N. Patel, H.K. Muduli & M.Sahoo, Advocates. (in I.A. Nos.5811 and 5898 of 2020)
	:	M/s. S. Das, B. Mohanty, C.K. Agarwal & A. Patnaik, Advocates. (in I.A. No.5779 of 2020)

Writ Petition (Civil) No.13853 of 2020

Dillip Kumar Ray ... Petitioner

-Versus-

State of Odisha & Others ... Opposite Parties

For Petitioner	:	In person.
For Opp. Parties	:	Mr. Chandrakanta Pradhan, Central Government Counsel (For Union of India)
	:	Mr. S. Satpathy, Advocate (For Shree Jagannath Temple Managing Committee)
	:	Mr. Ashok Kumar Parija, Advocate General

HONOURABLE THE CHIEF JUSTICE MR. MOHAMMAD RAFIQ
A N D
HONOURABLE MR. JUSTICE BISWAJIT MOHANTY

J U D G M E N T

09.06.2020

Per: Mohammad Rafiq, CJ.

These two writ petitions have been filed in the nature of Public Interest Litigation, raising the issue about the Car Festival of Lord Shree Jagannath at Puri, which is scheduled to take place on 23.06.2020. One of the above writ petitions i.e. W.P.(C) No.13853 of 2020, filed by one Dillip Kumar Ray, has been taken up on special mention today, along with W.P.(C) No. 12494 of 2020, already on board, after notice to learned counsel for the State-opposite parties.

2. The prayer in the first writ petition filed by Surendra Panigrahi-petitioner is that the Temple Managing Committee of Lord Shree Jagannath, Puri and other opposite parties-the State Government and other authorities, may be directed to postpone the Car Festival at Puri due to pandemic Coronavirus (COVID-19). Another writ petition has been filed with the prayer that Car Festival/Rath Yatra/Gundicha Yatra should be allowed to be held by strictly adhering to the conditions imposed in the guidelines issued by the Government of India, Ministry of Home Affairs, New Delhi vide Order No.40-3/2020-DM-I(A) dated 30.05.2020 (Annexure-B/5 to the Preliminary Counter Affidavit) and the order dated 01.06.2020 passed by the Government of Orissa, by maintaining social distancing and wearing mask. An additional prayer has been made that the Chariots/Car of Lords may be allowed to be pulled only by the heavy duty machineries rather than the men power.

3. We have heard learned counsel for petitioners, learned Advocate General and learned Counsel for the interveners.

4. Mr. S.K. Padhi, learned counsel for the petitioner in the first writ petition submitted that more than 10 lakhs people have always attended the Rath Yatra in the past. If the Car Festival is allowed to take place on 23.06.2020, it might attract lakhs of devotees this time also. The entire country including the State of Odisha is presently passing through a very critical phase due to the outbreak of pandemic Coronavirus. Such large gathering in and around Puri might

prove super spreader of Coronavirus; jeopardizing lives of thousands of people. Learned counsel submitted that the Government of India in its guidelines dated 30.05.2020, issued under the Disaster Management Act, 2005 has prohibited all kinds of social/political/sports/entertainment/academic/cultural/religious functions and other large congregations. It has directed the State Government not to dilute these guidelines in any manner. All the District Magistrates have been asked to strictly enforce the same. Any person violating these measures will be liable to be proceeded against as per the provisions of Sections 51 to 60 of the Disaster Management Act, besides legal action under Section 188 of the IPC, and other legal provisions.

5. Mr. Dillip Kumar Ray, the petitioner in person in W.P.(C) No.13853 of 2020, has also expressed similar apprehensions as have been voiced by learned counsel for the petitioner in the other writ petition. He submitted that if the State authorities are permitted to hold the Rath Yatra, they should be mandated to strictly adhere to the guidelines issued by the Government of India and the State Government, for maintaining social distancing and allow only limited number of Sebayat/Daitapati, priests and police personnel to participate, who should be mandated to wear masks. His additional submission is that rather than pulling manually, which might require seven to eight hundred persons, the Chariots/Car should be pulled with the help of heavy duty machineries so that involvement of such large number of persons in pulling the Chariots/ Car can be avoided.

6. Learned Advocate General submits that in view of the total lockdown imposed by the Central Government, the State Government vide its letter dated 06.05.2020 approached the Government of India, seeking permission for construction of Rathas as well as conduct of Rath Yatra. The Ministry of Home Affairs, Government of India vide its letter dated 07.05.2020 (Annexure-A/5 to the preliminary counter affidavit) addressed to the Chief Secretary of the State, while permitting construction of the Ratha to be undertaken in the Ratha-Khala, directed that no religious congregations shall be allowed to take place in the Ratha-khala and complete segregation should be ensured and the guidelines issued by the MHA and the National Directives for Covid-19 Management should be compulsorily adhered to. Attention of the Court in particular is invited towards para-4 of the aforesaid letter, wherein it has been stated that decision regarding holding of Ratha Yatra should be taken by the State Government, keeping in view the conditions prevailing at that point of time. Learned Advocate General submits that sub-clause (iv) of Phase III in Clause 1 of the latest guidelines dated 30.05.2020 (Annexure-B/5 to the preliminary counter affidavit) issued by the Central Government for Phased Re-opening (Unlock 1), categorically provides that dates for re-starting certain activities with regard to the social/ political/ sports/ entertainment/ academic/ cultural/ religious functions and other large congregations shall be decided based on the assessment of the situation. Clause 5 of the said guidelines further provides that the State Government, based on its assessment of the situation, may

prohibit certain activities even outside the Containment zones, or impose such restrictions as it may deem necessary. Clause 9 of the said guidelines directs that the State/UT Governments shall not dilute these guidelines issued under the Disaster Management Act in any manner. Learned Advocate General submits that this however does not mean that the State Government cannot enforce further restrictions on the basis of evaluation of the situation. He further submits that the State Government has also issued an order dated 01.06.2020 (Annexure-C/5 to the preliminary counter affidavit) containing these and many other guidelines. In sub-clause (v) of Clause 3 of the said order, it has been provided that Social/political/sports/entertainment/academic /cultural/religious functions and other large congregations will continue to remain closed till 30.06.2020.

7. Learned Advocate General submits that the first case of Coronavirus in State of Odisha was reported on 16.03.2020. Thereafter, due to the pre-emptive measures taken by the State Government to tackle the spread of Coronavirus, there was no spurt of positive cases in the State. However there has been a steep increase in the number of positive cases on account of recent influx of the migrants and people coming from the outside the State. It is submitted that while on 30.03.2020, there were only 3 positive COVID-19 cases in the State, but the number increased to 143, 2104 and 2856 on 30.04.2020, 31.05.2020 and 07.06.2020 respectively. The situation on ground is thus changing everyday. As per the assessment of the

experts in the medical field, number of positive Coronavirus cases is likely to increase sharply in the months of June and July, before reaching a peak. Learned Advocate General submits that the number of positive case at Puri on 1st may, 2020 was only one but number of such cases in district Puri has increased drastically and as on 07.06.2020, it has reached 108. Keeping this in mind, Puri was classified as a high risk zone and therefore included in the 11 districts earmarked for weekend (Saturday and Sunday) shutdown vide order dated 01.06.2020.

8. Learned Advocate General relied on the decision of this Court dated 04.06.2020 in *W.P(C) No.13539 of 2020*, wherein the petitioner Jayanta Kumar Bal approached this Court with a prayer that State Authorities may be directed to allow him and the devotees/senior citizens/sevakas/people of Puri to have darshan of Lord Shree Jagannath on Shnana Purnima on 05.06.2020 outside the Meghanada Pacheri and further prayed to quash and set aside the Clause 3 (a) (i) of the order dated 01.06.2020 issued by the Government of Orissa, being contrary to the guidelines issued by the Government of India dated 30.05.2020. This Court, while considering the guidelines issued by the Central Government dated 30.5.2020 and the order of the State Government dated 01.06.2020, declined to interfere in the matter by holding that the State Government in having extended the restrictions upto 30.06.2020 with regard to the entry into the religious places/places of worship, appears to have taken into consideration the larger public interest. It was held that decision of

the State Government was in consonance with the guideline issued by the Central Government, aimed at preventing spread of Coronavirus. Learned Advocate General submits that the decision whether or not to allow the Rath Yatra shall be taken by the State Government only few days before the scheduled date i.e. 23.06.2020, on the basis of the situation then prevailing on ground. Learned Advocate General drew the attention of the Court towards such specific stand of the Government in para 18 of its counter affidavit.

9. We have given our thoughtful consideration to rival submissions and examined the material on record.

10. Corona Virus disease, which has now come to be known as COVID-19, is caused by Novel Corona Virus. This was first detected in Wuhan city of Hubei province of China sometime in December 2019. This virus rapidly spread across the world—in and around 167 countries including India by mid of March 2020, as a result of which, the World Health Organization declared this as a pandemic. The Government of India comprehending the gravity of the problem invoked the Disaster Management Act 2005 (for short, the Act), for management of the disaster, where in the "disaster" has been defined in 2(d), where "disaster management" has been defined in 2(e) of the Act to mean "a continuous and integrated process of planning, organising, coordinating and implementing measures which are necessary or expedient for—(i) prevention of danger or threat of any disaster; (ii) mitigation or reduction of risk of any disaster or its

severity or consequences; (iii) capacity-building; (iv) preparedness to deal with any disaster; (v) prompt response to any threatening disaster situation or disaster; (vi) assessing the severity or magnitude of effects of any disaster; (vii) evacuation, rescue and relief; (viii) rehabilitation and reconstruction."

11. Section 3 of the Act envisages establishment of National Disaster Management Authority, headed by Prime Minister of the Country, as its *ex officio* Chairperson. Similarly, Section 14 of the Act provides for establishment of the State Disaster Management Authority with the Chief Minister of the State, as its *ex officio* Chairperson. The power and functions of National Authority has been enumerated under Section 6 of the Act, which includes the power for laying down the policies, plans and guidelines for disaster management; approving the National Plan; laying down guidelines to be followed by the different Ministries or Departments of the Government of India for the purpose of integrating the measures for prevention of disaster or the mitigation of its effects in their development plans and projects; and take such other measures for the prevention of disaster, or the mitigation, or preparedness and capacity building for dealing with the threatening disaster situation etc. Section 18 of the Act enumerates the powers and functions of the State Authority, which includes the power to lay down the State Disaster Management Policy; approve the State Plan in accordance with the guidelines laid down by the National Authority; approve the disaster management plans prepared by the departments of the Government of

the State; lay down guidelines to be followed by the departments of the Government of the State for the purposes of integration of measures for prevention of disasters and mitigation in their development plans and projects and provide necessary technical assistance therefor; and review the measures being taken for mitigation, capacity building and preparedness by the departments of the Government of the State and issue such guidelines as may be necessary.

12. While the National Plan has been separately defined in Section 11 of the Act, Section 23 stipulates the State Disaster Management Plan for the State, which may include the vulnerability of different parts of the State to different forms of disasters; the measures to be adopted for prevention and mitigation of disasters; the manner in which the mitigation measures shall be integrated with the development plans and projects; the capacity-building and preparedness measures to be taken; the roles and responsibilities of different Departments of the Government of the State in responding to any threatening disaster situation or disaster.

13. The National Disaster Management Authority while invoking the power under Section 6(2)(i) of the Act issued an order dated 24.03.2020, directing the Ministries/Departments of Government of India, and the State/Union Territory Governments and State/Union Territory Authorities to take effective measures to prevent the spread of COVID-19 in the country. As a consequence, the Ministry of Home Affairs, (MHA) issued an order dated 24.03.2020

under Section 10(2)(*l*) of the Act, imposing lockdown and directing all concerned to take effective measures for ensuring social distancing so as to prevent the spread of COVID-19 in the country. This order remained in force in all parts of the country for a period of 21 days with effect from 25.03.2020. National Lockdown was then extended further by order of the MHA dated 14.04.2020 upto 30.04.2020 and thereafter, by order dated 01.05.2020, it was extended upto 17.05.2020. A fresh order was then issued by the MHA on 17.05.2020 extending the lockdown measures so as to contain the spread of COVID-19 in the country for a period upto 31.05.2020. It is in continuation thereof that the MHA has now issued fresh directives extending the lockdown in the containment zones by order dated 30.05.2020, however named as Unlock-1, with certain prohibited activities being allowed to be reopened in phased manner in areas outside the containment zones.

14. The State Government of Odisha vide notification date 13th March, 2020 invoked the Epidemic Diseases Act, 1897 and the Code of Criminal Procedure to declare Coronavirus (COVID-19) a disaster. It also imposed restrictions on all kind of congregations so as to ensure “social distancing” for containing the spread of COVID-19. In fact, the Government of Odisha vide notification dated 8th April, 2020 issued Ordinance No.1 of 2020 for incorporating state amendments in the Epidemic Diseases Act, 1897 to make contravention or disobedience of any order or regulation made thereunder an offence punishable with imprisonment for a term which

may extend to two years or with fine which may extend up to ten thousand rupees or with both.

15. Perusal of the letter of the Central Government dated 07.05.2020 addressed to the Chief Secretary of the Government of Odisha, indicates that permission for construction of the Ratha in the Ratha-Khala was granted by imposing certain conditions but leaving the decision about holding of the Rath Yatra entirely to the discretion of the State Government, which would be evident from the following excerpts thereof:

> *"3. The undersigned is directed to convey that the activity of Ratha construction is allowed to be undertaken in the Ratha-khala, which is situated on both sides of the Grand Road in front of the Temple Office and Sri Nahar (Palace), subject to the following conditions being fulfilled:*
>
> *a) No religious congregation takes place in the Ratha-khala. Complete segregation of Ratha-khala should be ensured.*
>
> *b) The new guidelines on lockdown measures issued by MHA on 1st May, 2020, including the National Directives for Covid-19 Management, should be compulsorily adhered to.*
>
> *4. However, the decisions regarding holding of Ratha Yatra be taken by the State Government keeping in view the conditions prevailing at that point of time."*

16. Sub-clause (iv) of Phase III of the latest guidelines dated 30.05.2020 issued by the Central Government categorically provides that decisions to re-start the activities in the nature of social/political/sports/entertainment/academic/cultural/religious functions and other large congregations shall be taken based on the

assessment of the situation. Additionally, the Central Government in Clause-5 of the said guidelines provides that "State/UTs, based on their assessment of the situation, may prohibit certain activities outside the Containment zones, or impose such restrictions as deemed necessary". In Clause-9 of the aforesaid guidelines, the State/UT Governments have been mandated not to dilute any of these guidelines, which have been issued under the Disaster Management Act and all the District Magistrates have been required to strictly enforce these measures. Clause 10 has made violation of any of these measures punishable as per the provisions of Sections 51 to 60 of the Disaster Management Act besides under Section 188 of the IPC.

17. The State Government, on its part having objectively assessed the situation on ground, has imposed certain additional and further restrictions in its order dated 01.06.2020, which would be evident from the Clause 3 thereof:-

> *"**3. Graded re-opening of areas outside the Containment Zones***
> *In areas outside Containment Zones, activities will be regulated as below:*
>
> *a. The following establishments/activities will continue to remain closed till 30th June, 2020:*
> *(i) Religious places/places of worship for public.*
> *(ii) Shopping malls*
> *(iii) International air travel of passengers, except as permitted by MHA.*
> *(iv) Cinema halls, gymnasiums, swimming pools, entertainment parks, theatres, bars and auditoriums, assembly halls and similar places.*
> *(v) Social/political/sports/entertainment/academic/cultural/religious functions and other large congregations.*

b. Hotels will be allowed to operate up to 30% capacity. Restaurant service will be open only for in-house guests.

c. Restaurants and Hotels are permitted for home delivery/takeaways of food.

d. Schools, colleges, other educational/training/ coaching institutions, etc. will remain closed till 31st July, 2020."

18. No doubt, the various preventive measures introduced by the Central Government in the guidelines issued under the direction of the National Disaster Management Authority, in the state of Odisha, have been implemented and enforced. But the power of the State Government in issuing further and additional guidelines, which do not have the effect of diluting the measures introduced in the guidelines of the Central Government cannot be denied. The State Government is equally competent to prescribe and enforce such additional and further measures as it may deem necessary on the recommendation of the State Disaster Management Authorities in the State Plan, as per the provision of Sections 14 to 24 in Chapter III of the Act.

19. This Court was approached by one Jayanta Kumar Bal in *W.P(C) No.13539 of 2020* questioning the competence of the State Government; in particular about continuing restrictions on entry into places of worship for public even beyond 08.06.2020, upto 30.6.2020 and praying to allow him and other devotees/senior citizens/sevakas/people of Puri to have darshan of Lord Shree Jagannath on Shnana Purnima on 05.06.2020. Repelling the contention, this Court upheld the aforesaid guidelines holding thus:-

> "*5. Having heard learned Senior Counsel for the petitioner and learned Advocate General for the State-opposite parties and taking note of the rival submissions, so far as first part of the prayer is concerned keeping the second part open, and considering the guideline issued by the Central Government and the order dated 01.06.2020 passed by the State Government, we are not inclined to hold that the impugned order is in any way opposed to public interest. On the contrary, in our view, the order of the State Government appears to have been passed taking consideration of larger public interest and in consonance with the guideline issued by the Central Government in order to prevent spreading of Coronavirus. This Court would be loath to interfere with the decision of the State Government, which appears to be based on objective evaluation of situation, as in the opinion of this Court, such matters are best left to the discretion of the executive."*

20. What emerges from the submissions made by learned Advocate General appearing for the State, especially in view of the stand taken by the State Government in Para 18 of the counter affidavit is that State Government is yet to take a decision on the question of holding of Rath Yatra. Para-18 supra is for the facility of reference reproduced hereunder:-

> "18. That in view of the aforesaid and keeping in mind the deteriorating situation pertaining to the spread of COVID-19 virus in the State of Odisha, the State Government is constantly monitoring the situation and any decision with regard to the holding of the Ratha Yatra festival will be taken on the basis of the objective situation of the pandemic as on the relevant date and keeping in mind the interest of the public at large."

In view of the above, it is evident that the State Government is fully cognizant of the deteriorating situation about the spread of Coronavirus in the State. It is constantly monitoring such situation and will take a decision with regard to holding or otherwise, of the Ratha Yatra, on the basis of objective evaluation of the ground

situation at an appropriate time, prior to the scheduled date i.e. few days before 23.6.2020, keeping in view safety, security and welfare of the State.

21. While therefore not issuing any mandamus as prayed for, this Court is inclined to hold that it is up to the State Government to decide whether or not to allow the Rath Yatra on 23.6.2020, depending on the situation then prevalent on the ground about the spread of Coronavirus. If however any such decision is eventually taken, the State Government shall ensure strict adherence to the directives issued by the Government of India in Clause 3 of their letter dated 07.05.2020; with regard to the adherence to the lockdown measures issued by the Ministry of Home Affairs, Government of India in their Guidelines dated 30.5.2020 and also the National Directives for Covid-19 Management. The State Government shall also ensure strict adherence to its own order dated 01.6.2020 containing additional and further guidelines. As regards the other prayer that the Chariots/Car should be allowed to be pulled manually or mechanically, we are inclined to observe that deploying heavy duty machineries or any other means like elephants, than the men power, for pulling the Chariots/Rath, would obviously obviate the necessity of involving large number of persons, which number could be in many hundreds. It is therefore directed that this aspect should be duly considered by the State Government while taking a decision for holding Rath Yatra, consistent with the guidelines issued by the Central Government and the State Government.

22. With the above observations, both the writ petitions are disposed of.

As Lock-down period is continuing for COVID-19, learned counsel for the petitioner may utilize the soft copy of this judgment available in the High Court's official website or print out thereof at par with certified copies in the manner prescribed, vide Court's Notice No.4587 dated 25.03.2020.

(BISWAJIT MOHANTY)
JUDGE

(MOHAMMAD RAFIQ)
CHIEF JUSTICE

//M. Panda//P.A.

2. Guidelines for care and management of captive elephants

Ministry of Environment & Forests

Project Elephant

No 9-5/2003-PE dated 8-1-08

To

The CWLW (All States/UTs)

Sub - Guidelines for care and management of captive elephants

Sir

Asian elephant has been accorded highest protection by listing them in Schedule I of the Wildlife (Protection) Act 1972 , Ministry is quite concerned about its protection , care and management . It may be recalled that Ministry had constituted an expert committee under the Chairmanship of Mr S C Dey , EX ADG (WL) in the year 2003 to study the status of elephants in India . The Committee submitted its report in 2004. The Committee also has given several recommendations for care and general welfare of captive elephants. Ministry vide letter no 9-5/03-PE dated 1.6.2005 had requested all CWLWs for action on following points:

1. Need for framing special rule / guidelines for management & care of captive elephants on lines of Kerala.
2. Need for improving training facility for mahouts & elephants .
3. Need for improving working condition of mahouts including wages / salary.
4. Need for better vet care for captive elephants.
5. Need for enforcing the legal provisions particularly for ownership and prevention of cruelty.

However , Ministry has not received any feedback from any of the State / UT of the action initiated on above.

The matter was discussed in detail with CWLWs of the States having major population of captive elephant and it was decided that till states formulate their own Rules , Central government should consider issuing a guideline laying down norms for transportation , housing , feed , vets care and other norms for the states to follow . Accordingly a detailed guidelines for the care and management of captive elephants is being circulated . It is requested to ensure its implementation in letter and spirit.

Encl. As above

Yours faithfully

(A N Prasad)
IGF & Director (PE)

Copy to – IGF (WL) / MS , CZA/ Director , WII , Dehradun for information and necessary action.

Guidelines for care and management of captive elephants

Ownership certificate

1.All States / UTs would carry out a fresh survey of the captive elephants in their territory within a period of six months and report the number to the Ministry. All the captive elephants shall be microchipped for which chips have been provided in adequate numbers to the states /UTs. Fresh ownership certificate should be issued in the form annexed for a period of five years and should be renewed every five years in case there is no violation of the norms to be followed.

2.Illegal elephants i.e those which have not been declared under the declaration of the Wild life Stock Rules 2003 or are found without valid documents will be confiscated.

3.It would be mandatory for the owners to declare in writing in advance to the nearest Divisional Forest Officer or to the authorized officer by the state government of the pregnancy of the female elephants in order to get fresh ownership certificate of the calf . The certificate would be issued to the calves of the legal cows only after physically ascertaining that they are genuine offspring or after getting the DNA test done.

Transportation of elephants

1.For transportation of elephants , necessary permission from CWLW or any officer authorized by the government in this behalf shall be obtained as per section 48 A of the WP Act 1972.

2.A valid health certificate from a veterinary doctor to the effect that the elephant is fit to travel by raid or rail , as the case may be , and is not showing any sign of infectious or contagious disease shall be obtained .

3.In the absence of such certificate , CWLW shall not give permission for transport.

4.Permission , if any , should be given for transport to a particular destination and for a fixed period. It would be incumbent for the owner to bring back the elephant to the place of residence within this period .

5.Before the issue of transport permit , CWLW or the authority issuing the permit would obtain no objection from the CWLW of the state where it is to be transported about the availability of the adequate housing facility at the place where it is to be kept .

6.It will be mandatory for the owner to inform the CWLW of the state within 30 days where it has been transported .

7.Except for return journey , no permission for the further onward transport of the elephant to other states will be given by the CWLW of the state where it is in transit .

8.In case any captive elephant is found in any state wiyhout valid transport certificate , it is laible for confiscation.

9.CWLWs may consider banning of captive elephants entry in Municipal limits under sec 40 (2) .

Norms and Standards for Transportation

(d) The elephant shall be properly fed and given water before loading;

(e) Necessary arrangements shall be made for feeding and watering the elephant en route;

(f) No elephant shall be made to walk for more than three hours at a stretch;

(g) While transporting elephants by walk during nights, two prominent reflectors shall be placed at the front and hind portion of the elephant;

(h) No elephant shall be made to walk more than 30 kms a day and any transportation for more than 50 kms shall be carried out in a vehicle;

(i) Trucks with length less than 12 feet shall not be used for carrying elephants except calves (height below of and 1.59 m)

(j) One truck shall not be used to carry more than two weaned calves (height below 1.50 m) or one elephant with one unweaned calf or one adult/sub-adult elephant (height above 1.51 m);

(k) At least 12 hour rest should be allowed to elephants for every 12 hours of journey by trucks.

(l) Cow elephants in advanced stage of pregnancy shall not be transported by trucks;

(m) While transporting elephants by rail, an ordinary goods wagon should not carry more than three adult elephants or six calves on broad gauge, or not mort ha two elephants or three calves on meter gauge, or not more than one adult elephant or two calves on narrow gauge;

(n) While transporting elephants by truck or train, care shall be taken to maintain constant speed avoiding jerks and sudden stops and reducing affects of shocks and jolts to the minimum;

(o) Each truck or wagon carrying elephant should have at least two attendant mahouts;

(p) Sedatives, if necessary, shall be used to control nervous or temperamental elephants only as prescribed by the veterinary doctor.

(q) Vehicle breakdown is one of the most common problem contributing to unsuccessful translocation . Therefore it should be ensured that vehicle is in order and a trained mechanic with tool must accompany the vehicle. Vehicle must have drainage facility to keep it dry and must have a water storage facility.

Housing of Elephants

(a) The owner shall provide a stable (tethering place) in a clean and healthy environment with sufficient shade to keep elephants during its rest period;

(b) Each elephant must be ensured a minimum floor area as specified below:-

(i) Weaned Calf (height below 1.50 m) 5m x 2.5m

(ii) Sub-adult elephant (height 1.50 m to 2.25 m) 7m x 3.5m

(iii) Adult elephant (height above 2.25 m) and

Cow elephant with unweaned calf 9m x 6m

(c) In the case of covered sheds, the height of the structure shall not be less than 5.5m;

(d) Corrugated iron sheets or asbestos when used for roofing of elephant stables shall be covered with cooling materials like gunny bags, grass, cadjan leaves etc.

Care of Elephant

(a) The mahout shall ensure that the elephant gets a thorough bath every day;

(b) If the elephant is found sick, injured, unduly stressed or pregnant the mahout shall report the condition to the owner, who in turn shall consult a Veterinary Doctor for providing treatment expeditiously;

(c) Routine examination including parasitic checks shall be carried out regularly and preventive medicines including vaccination be administered at such intervals as may be prescribed by the Veterinary Doctor;

(d) The owner shall arrange for medical check-up of the mahout responsible for upkeep of the elephant at least once in two years to ensure that they do not have any diseases, which may infect the elephant;

(e) The organizers of festivals where elephants are used shall submit in writing the programmes with details to the station house officer and the Range Officer having jurisdiction over the area, who in turn shall ensure the implementation of the provisions in these rules;

(f) The owner shall inform within 24 hours, to the Chief Wildlife Warden or the nearest forest officer, the cases of attack of anthrax, rinderpest, hemorrhagic scepticemia, surra or any other contagious diseases and shall follow the instructions issued by the authorities regarding the treatment of the animal or disposal of the carcass. The Chief Wildlife Warden or an officer authorized by him shall ensure proper veterinary assistance and advice;

(g) The owner shall obtain prior permission of the Chief Wildlife Warden or the officer authorized by him before undertaking distortions, sterilization, vasectomy, tubectomy or any other population control measures for the elephant and shall ensure the assistance of a competent veterinary doctor for these measures;

(h) The elephant showing symptoms of musth shall be got examined by a Veterinary Doctor;

(i) No drugs or intoxicants shall be used to suppress musth except on a written prescription by a Veterinary Doctor;

(j) The owner of the elephant shall ensure that in case of musth, the elephant is secured properly and does not become a hazard to the public at large;

(k) An elephant in musth shall not be put to any work;

(l) No owner shall put to work, any elephants having pregnancy of 12 months or above, or any cow elephant having a sucking calf of age below 6 months, or any elephant of height below 5 feet;

(m) No owner shall permit the use of nylon ropes or chains/hobbles with spikes or sharp edges for trying the elephants;

(n) Weight of the chains and hobbles shall commensurate with age and health of the elephant;

(o) No owner shall permit any type of harness which may expose the back or other sensitive organs of the elephant to pain and injury;

(p) No owner shall permit his elephant to be trained by a trainer who is not approved by the Chief Wildlife warden or the officer authorized by him for the purpose;

(q) The owner shall report within 24 hours, to the Chief Wildlife Warden or to the officer authorized by him, the death of an elephant and the tusks, if any, shall be declared within one week to the Chief Wildlife Warden for obtaining Ownership Certificate;

(r) The owner shall get the postmortem examination of the elephant done by a veterinary doctor and shall submit the report to the Chief Wildlife Warden or the officer authorized by him within 15 days of the death.

Feeding of Elephants

(a) The owner or the person who is managing the elephant on contract or the person who has taken the elephant for own purpose shall ensure timely supply of wholesome feed with variety in required quantity to each elephant. Green fodder shall be supplemented by ration as prescribed by veterinary doctor;

(b) The minimum feed supply for elephant shall be as follow:

Height of Elephant	Green Fodder
Below 1.59 m (weaned calf)	Not less than 100 kg
1.50 m to 1.80 m	Not less than 150 kg
1.81 m to 2.25 m	Not less than 200 kg

Above 2.25 m	Not less than 250 kg (or 5% of its body weight)

(c) Supply of sufficient quantity of succulent food to the elephant shall be ensured during hot climate;

(d) The owner or contactor or hirer of the elephant shall provide sufficient potable drinking water to the elephant, preferably from a river or any other soure of running water.

Work Load of Elephant

(a) The scale of load including gears, riders and materials for the elephant shall be as follow:

Height of elephant	Load
Below 1.50 m	Not to be used for carrying load.
1.50 m to 1.80 m	Not exceeding 150 kg (to carry only fodder and trainer)
1.81 m to 2.25 m	Not exceeding 200 kg
2.26 m to 2.55 m	Not exceeding 300 kg
Above 2.55 m	Not exceeding 400 kg

(b) The load scale shall be reduced by 50% in hilly or other difficult terrain;

(c) The elephants of height below 2.10 m shall not be deployed for logging operations;

(d) The elephants of height from 2.10 m to 2.25 m shall not be used for dragging timber logs exceeding 750 kg in weight;

(e) The elephants of height above 2.25 m shall not be engaged for dragging logs exceeding 1000 kg in weight;

(f) Ill-designed logging harness such as exposing elephants back bone and chest to

extreme strain and injuries, using tusks and jaws regularly for dragging timber logs,

timber hauling over steep areas or rocky areas etc. shall not be done.

Retirement of Elephants

(a) An elephant shall normally be allowed to retire from its work on attaining an age of 65 years;

(b) Healthy elephants above 65 years of age shall be allowed to be put to light work under proper health certificate from the veterinary doctor.

Records to be kept

(a) Every owner shall maintain the following records and registers in respect of the elephant in the form given in appendix-II and such records and registers shall be produced before the officers authorized by Government in this behalf for inspection at such time as may be called for.

(i) Vaccination record.

(ii) Disease and treatment record.

(iii) Movement register.

(iv) Feeding register.

(v) Work register.

Cutting Tusks

(a) The owner of the tusker shall apply for permission of the Chief Wildlife Warden or the officer authorized by him in this behalf, for cutting or shaping the tusk through a letter sent by registered post, including the location where it will be done and the name of the competent person who would perform the operation at least one month in advance;

(b) The Chief Wildlife Warden shall issue the permission within three weeks to carry out the operation in the presence of an officer not below the rank of Forest Range Officer or Forest Veterinary Officer or Assistant Forest Veterinary Officer as instructed by the Chief Wildlife Warden;

(c) The authorized officer shall report to the Chief Wildlife Warden, the details of the cut portion such as, length and weight of the tusk;

(d) In case permission is not granted, the owner shall be intimated of the reason for rejecting the request in writing;

(e) The Chief Wildlife Warden, based on a written request with the details shall issue permit to the owner for keeping the cut tusks in accordance with the provisions of the Act.

Acts which are tantamount to cruelty to elephants :- The following acts shall be considered as acts of cruelty to elephant and is prohibited:-

(a) beating, kicking, over-driving, over-loading, torturing or treating any elephant so as to subject to it to unnecessary pain or suffering, or being an owner permitting, any elephant to be so treated;

(b) employing in any work or labour or for any purpose, any elephant which by reason of its age or disease, informity, wound , sore or other cause, if unfit to be so employed, or being owner permitting any such elephant to be employed;

(c) willfully and unreasonably administering any injurious drug or injurious substance to an elephant or uses drugs or intoxicants to control elephants particularly to suppress musth without proper veterinary advice;

(d) conveying or carrying whether in or upon any vehicle or not, an elephant, in such a manner or position as to subject it to unnecessary pain or suffering or cause accident;

(e) keeping or confining an elephant, in any cage or receptacle, which does not measure the specification given in rule 4;

(f) keeping for unreasonable time, an elephant chained or tethered upon an unreasonable short or unreasonably heavy chain or cord;

(g) using an elephant for drawing any vehicle or carrying any load, more than nine hours a day or for more than five hours continuously without a break or rest for the elephant or exposes the elephant to hot climatic conditions without ensuring enough succulent food and electrolytes;

(h) failing to provide an elephant, with sufficient food, drinking water or shelter;

(i) abandoning an elephant in circumstances, which will render it to suffer pain by reason of starvation or thirst;

(j) offering for sale any elephant, which is suffering from pain by reason of mutilation, starvation, thirst, over-crowding or other ill treatment;

(k) not providing adequate veterinary care to a sick, injured or pregnant elephant;

(l) cutting the tusks of a bull elephant too short so as to expose horn cord/pulp;

(m) forcibly weaning away an elephant calf below 2 years of age from its mother;

(n) using heavy chains and hobbles with spikes or sharp edges or barbed wires for tying elephants;

(o) using "peti" (belly band) on cow elephants in advanced stage of pregnancy;

(p) using pad and Nundah of improper size on working elephant exposing its spinal cord to injuries;

(q) marching a sick, injured to or pregnant elephant or a young calf over a very long distances or for a long duration at a stretch;

(r) marching an elephant over tarred roads or otherwise, during hottest period of the day and for a long duration at a stretch without rest for religious or any other purpose;

(s) transporting elephants on trucks of inadequate size or trucks with uneven floor, or tying them in an improper manner-subjecting them to severe jerks during jouney by truck;

(t) transporting elephants in trucks for over 12 hours at astretch;

(u) transporting elephants through any conveyance without making arrangement for adequate fodder and drinking water during the journey;

(v) carrying load on an elephant without proper pad;

(w) making an elephant carry load unevenly balanced on its back;

(x) making the elephant to stand in scorching sun for long duration, or put the ceremonial gears or decoration for unreasonably long duration, or bursts crackers from or near the elephants for ceremonial purpose;

(y) using an elephant in such a manner so as to cause any injury, over-stress or strain to the elephant for tourism purpose;

(z) using an elephant for sports and games such as tug-of-war, foot ball etc. in such a manner so as to cause over stress or strain to the elephant.

APPENDIX II

FORM FOR CERTIFICATE OF FITNESS TO TRAVEL ELEPHANTS

(This certificate should be completed and signed by a Veterinary Doctor)

Date and Time of Examination

Number of Elephants

Name of Elephants

Age/Sex

Number of Cages

1. That, at the request of (consignor).....I examined the above mentioned elephants in their traveling cages not more than 12 hours before their departure.

2. That each elephant appeared to be in a fit condition to travel from the .....

.....area to..... by road/ rail and is not showing any signs of infections or contagious diseases.

3. That no cow elephant appeared to be under advanced stage of pregnancy.

4. That the elephants were adequately fed and watered for the purpose of the journey.

5. That the elephants have been vaccinated.

(a) Type of vaccine/s

(b) Date of vaccination/s

Signed

Address

Qualifications

Place:

Date:

APPENDIX III

FORMS OF RECORDS AND REGISTERS TO BE KEPT

1. Vaccination Record

(a) Name of the Elephant:

(b) Sex:

(c) Age:

Date of Vaccination	Name of Disease	Due date for next Vaccination	Signature of the Veterinary Surgeon

2. Disease and Treatment Record

(a) Name of the Elephant:

(b) Sex:

(c) Age:

Date of Treatment	History by Veterinary	Description	Diagnosis	Treatment	Prevention Measure	Signature of Veterinary

Surgeon	Surgeon

3. Movement Register

(a) Name of the Elephant:
(b) Sex:
(c) Age

Date	Place to Move		Time		Signature of the Mahout
	Starting	Ending	Starting	Ending	

4. Feeding Register

(a) Name of the Elephant:
(b) Sex:
(c) Age:
(d) Ration prescribed by the Veterinary Surgeon:

Date	Type of Food	Quantity given	Signature of Mahout

5. Work Register

(a) Name of the Elephant:
(b) Sex:
(c) Age:
(d) Admissible quantum of work:

Date and Weather	Type of Work	Duration	Signature of Mahout
		From – To	

STUDIES/RESEARCH

During the 10th Plan following research / studies were sanctioned and completed under the PE Scheme:

1. Anatomical studies on the Asian elephants of Assam – Assam Agriculture University (AAU) , Pl- Dr Munmun Sharma – period 2003-04 to Mar 20008. Cost Rs 4 lakh.
2. Preparation of health care and management protocol for the captive elephants of NE India – AAU , Pl – Dr K K Sharma – period 2003-04 to Sept 2007. Cost Rs 4.50 lakh.
3. Development of long awned rice varieties and paddy storage structure – CRRI , ICAR , Cuttack , Pl – Dr B C Patra – period 2003-04 to Sept 2007. Cost Rs 7.12 lakh .

Reports of the Studies / workshop

General and Comparative Endocrinology 201 (2014) 37–44

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Non-invasive assessment of reproductive status and stress in captive Asian elephants in three south Indian zoos

Vinod Kumar [a], Vivekananda Palugulla Reddy [b], Adiseshu Kokkiligadda [a], Sisinthy Shivaji [a], Govindhaswamy Umapathy [a,¤]

[a] Laboratory for the Conservation of Endangered Species (LaCONES), CSIR-Centre for Cellular and Molecular Biology, Hyderabad, India
[b] Indian Institute of Science Education and Research, Pune, India

ARTICLE INFO

Article history:
Received 30 September 2013
Revised 7 March 2014
Accepted 13 March 2014
Available online 1 April 2014

Keywords:
Asian elephant
Progestogen
Androgen
Glucocorticoids
Stress
Musth

ABSTRACT

Asian elephants in captivity need immediate attention to be bred so as to meet the increasing demand for captive elephants and to overcome the dependence on supplementing the captive stock with wild animals. Unfortunately, captive breeding programs across the globe have met with limited success and therefore more effort is needed to improve breeding in captivity. Endocrine profiling of reproductive hormones (progestagens and androgens) and the stress hormone (glucocorticoids) could facilitate better management and breeding strategies. In the present study, we investigated reproductive and stress physiology of 12 captive Asian elephants for 10–27 months using a non-invasive method based on steroid analysis of 1700 elephant dung samples. Most of the elephants were cycling regularly. Males during musth showed increased fecal androgen metabolite concentrations and exhibited a slight increase in fecal glucocorticoid metabolite levels. Elephants used in public festivals and processions showed significantly increased in faecal glucocorticoid metabolite levels. The results indicate that captive elephants require periodic health care, better husbandry practices and scientific management for sustainable captive population.

© 2014 Elsevier Inc. All rights reserved.

1. Introduction

Asian Elephant (*Elephas maximus*) is an endangered species (IUCN, 2012) with both *ex situ* and *in situ* populations decreasing at an alarming rate. Its survival is under threat because of continuous poaching and loss of habitat and corridors due to rapid habitat fragmentation. The captive Asian elephants constitute 22–30% of the total Asian elephant population (Lair, 1997; Sukumar, 2003) and are an indispensable workforce for forest departments. Elephants are a big tourist attraction and frequently used in religious processions. In India, captive elephants are normally managed by traditional knowledge and skills of mahouts. But, over the years, the quality of mahouts available has declined due to low monetary and improper welfare measures (MoEF 2004; Vanitha et al., 2009) thus impinging on the management of captive elephants (Vanitha et al., 2009). The problem is further compounded by the fact that captive breeding in elephants is not very successful (Rees, 2003), as e.g. less than 20% of Asian and 10% of African elephants of reproductive age have given birth in captivity in North American zoos (North American Regional Studbook, 2010; Brown et al., 2004). As a result, captive populations are regularly supplemented with elephants caught in the wild, adding further pressure on the struggling wild population (Sukumar, 1989). The limited success in captive breeding of elephants seems amongst others attributed to ovarian inactivity and acyclicity due to reproductive pathologies, neoplasias, etc. (Millspaugh and Washburn, 2004; Keay et al., 2006).

Studies on the reproductive physiology of female African (Wasser et al., 1996; and Asian elephants (Brown et al., 2004, 2009; Brown, 2000; Slade-Cain et al., 2008; Thitaram et al., 2008; Ghosal et al., 2011) have indicated that female captive elephants exhibit a long oestrous cycle of 14–17 weeks, with a 4–8 week follicular phase and an 8–10 week luteal phase (Brown, 2000; Slade-Cain et al., 2008; Hodges, 1998; Brown et al., 1999). Thus, routine endocrine monitoring can be viewed as a valuable tool to make decisions about the reproductive management of elephants.

Male elephants mature at the age of 15–20 years and periodically enter into a sexually active mode known as "musth". During musth they spend most of their time searching for females and less time in feeding resulting in significant weight loss (Poole and Moss, 1981). In African elephants, the association between glucocorticoids and musth is not clearly understood (Ganswindt et al.,

¤ Corresponding author. Address: LaCONES, CSIR-Centre for Cellular and Molecular Biology (CCMB), Uppal Road, Hyderabad 500007, India. Fax: +91 40 27160311.
E-mail address: guma@ccmb.res.in (G. Umapathy).

http://dx.doi.org/10.1016/j.ygcen.2014.03.024
0016-6480/© 2014 Elsevier Inc. All rights reserved.

2003), while other studies in Asian elephants have shown significant relationship between testosterone and cortisol levels in blood during musth (Brown et al., 2007; Rasmussen et al., 1984). Thus, understanding the changes involved during musth could immensely help in management and breeding of elephants in captivity.

With the advent of non-invasive hormone analysis using dung samples, it is indeed possible to monitor faecal progestogen, glucocorticoid and androgen metabolites levels to assess the reproductive cycle and stress of captive male and female elephants both on a short and long-term basis without collecting blood samples. In the present study, we examine the reproductive (progesterone, testosterone) and stress (cortisol) status of 12 captive Asian elephants with reference to different husbandry practices and body conditions in three south Indian zoos. More specifically, we investigate (i) whether reproductive cycle in females, (ii) stress levels in both the males and females, and (iii) musth in males are affected by different husbandry practices in three south Indian zoos.

2. Materials and methods

2.1. Sample collection

About 1700 samples were collected from 12 elephants (four males and eight females) from three south Indian zoological parks over a period of 280–800 days (Table 1). Elephant dung samples were collected twice a week from Nehru zoological park, Hyderabad (3 females and 1 male), Sri Chamarajendra zoological garden, Mysore (3 females and 1 male) and Sri Venkateshwara zoological park, Tirupati (2 females and 2 males), in the morning hours between 7 and 8 am in 50 ml plastic tubes (Table 1). Dung samples were also collected daily (for 10 days before and after public procession) from one female, used for festivals (Vanaja) was housed at Hyderabad zoo. The duration of sample collection was 27 months (July 2010–September 2012) for Hyderabad zoo, 14 months (July 2011–August 2012) for Mysore zoo and 10 months (May 2012–February 2013) for Tirupati zoo.

2.2. Sample storage

Samples collected in Hyderabad zoo were immediately frozen after collection and stored at −30 °C whereas Mysore and Tirupati zoo samples were stored in 80% methanol and brought to the lab within a week for further analysis. Methanol stored samples were further extracted within a week after collection to avoid variation in hormone concentrations due to long-term storage of samples (>3 months) as reported by Hunt and Wasser (2003). To examine different storage conditions, the dung samples were pooled from different animals and mixed thoroughly to ensure the hormone distributed properly before making subset of samples ($n = 7$, each of 5 samples) for different day storage and extraction analysis (Hunt and Wasser, 2003). Faecal hormone metabolite concentration did not vary significantly for 30 days of storage in methanol (K–W $X^2 = 9.13$, $P = 0.17$, $n = 35$, for faecal progestogen and K–W $X^2 = 9.48$, $P = 0.15$, $n = 35$. for faecal glucocorticoids, respectively). Furthermore we did not find significant difference in faecal steroid metabolites concentrations between samples directly stored in −30° C and those preserved in methanol in 30 days (faecal progestogen M–W Test, $U = 97$ $P = 0.10$, $n = 10$; faecal glucocorticoid M–W Test, $U = 6.5$, $P = 0.22$, $n = 10$; Fig. 1a and b).

2.3. Study animals and husbandry

The age of the elephants ranged from 9 to 58 years (Table 1). In Mysore zoo, both male and females were kept together in a large enclosure from 8 am to 5 pm unchained and rest of the time they were individually chained in a shed. In Hyderabad zoo, two females (Asha and Jamuna) were allowed to roam freely in a large open enclosure from 10 am to 4 pm and subsequently chained for the rest of the time in a shed, which also housed two other elephants, Vanaja, a female and Vijay, a male. Vanaja was taken for rounds inside the zoo a few days prior to use in public processions or festivals by *mahouts*. The male in Hyderabad zoo was always chained due to its aggressive nature and was not allowed to interact with other individuals. In Tirupati zoo, the elephants were individually released between 10 am and 4 pm, on alternative days, into the adjacent reserve forest with long chains to forage and for the rest of the day they were kept chained in a shed with only visual contact with males.

2.4. Musth and behavioural observations

Musth conditions in bulls were identified when temporal gland secretion, urine dribbling and increased aggressive behaviour were all observed for two consecutive observations in three days time (Ganswindt et al., 2005a; Poole, 1987). The above information, participation in public procession, injury and other activities were

Table 1
Details of age/sex, body condition, duration of sample collection, cyclicity status and faecal glucocorticoid metabolite concentrations in captive Asian elephants from three south Indian zoos.

S No	Zoo	Name of elephant	Sex	Age as on July 2013 (years)	Body condition grade*	Duration of sample collection (days)	Female cyclicity status	Mean faecal glucocorticoid metabolite concentration# (ng/g dry weight)	Faecal glucocorticoid metabolite concentration range (ng/g dry weight)
1	Hyderabad	Vijay	Male	29	4	800	–	3.86	0.20–16.80
2	Hyderabad	Jamuna	Female	39	8	800	Yes	4.10	1.00–12.00
3	Hyderabad	Asha	Female	40	6	800	Yes/ irregular	5.00	1.00–79.00
4	Hyderabad	Vanaja	Female	42	6	800	Yes/ irregular	5.24	1.21–49.25
5	Mysore	Rama	Male	18	7	385	–	3.19	0.32–8.62
6	Mysore	Airavathi	Female	9	8	385	Yes	3.84	0.75–15.00
7	Mysore	Gajalakshmi	Female	33	9	385	Yes	2.98	0.20–7.82
8	Mysore	Padmavathi	Female	58	8	385	No	2.54	0.27–7.10
9	Tirupati	Venkanna	Male	32	8	284	–	3.40	2.10–7.90
10	Tirupati	Vinayaka	Male	40	8	280	–	9.31	3.40–20.40
11	Tirupati	Rani	Female	57	7	280	Yes	8.31	3.60–21.70
12	Tirupati	Padmavati	Female	12	8	280	Yes	9.41	4.80–75.00

* Body condition rating was done using criteria described in Wemmer et al. (2006).
\# Excluding values during musth, injury and public procession.

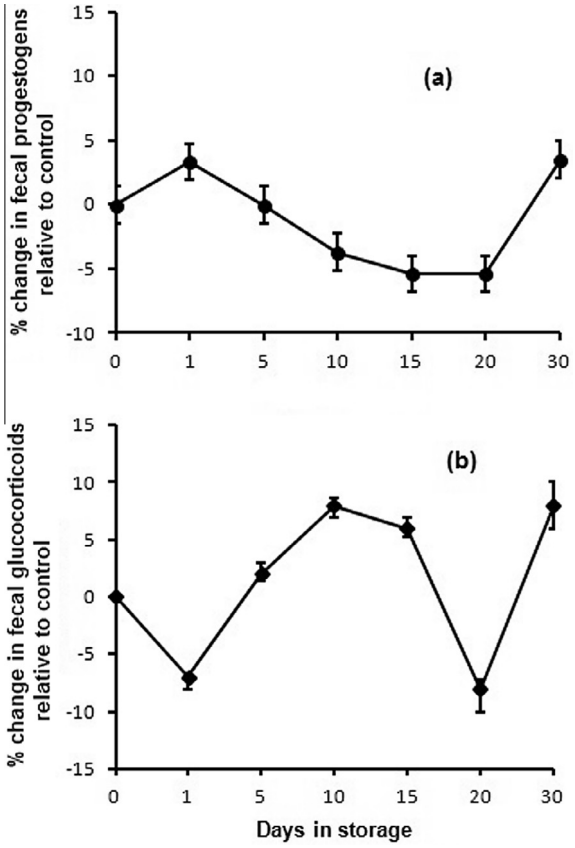


Fig. 1. Effect of storage in methanol at room temperature on faecal progestogen (a) and glucocorticoid (b) metabolite concentrations. Data (mean ± SEM, $n = 5$ for each point) are expressed as percentage change relative to the control (stored at −30 °C).

recorded *ad libitum* on alternative days on a data sheet by animal keeper during the study period (Altmann, 1974). Body condition rating was assessed by the same person (AK) for all the elephants as per the previous report (Wemmer et al., 2006).

2.5. Extraction of steroid metabolites

The dung samples were extracted according to the procedure described earlier (Ganswindt et al., 2005a) with some modifications. The samples stored at −30 °C and methanol preserved samples were dried at 70 °C overnight. The dried samples were pulverized, sieved and 0.2 g of the resulting faecal powder sample was transferred to 15 ml falcon tubes to which 5 ml of 80% methanol was added, and the sample vortexed for 20 min. After this, samples were centrifuged at 3300*g* followed by collection of supernatant in 5 ml plastic cryovials, which were stored at −30 °C until further assay. Extraction efficiency was determined by adding known amount of ^{3}H labelled steroids (progesterone, cortisol, and testosterone, $n = 10$ for each) to faecal sample before extraction (Umapathy et al., 2013). Extraction efficiency was 80.16 ± 2.3 for progesterone, 76.23 ± 3.1 for cortisol and 86.45 ± 4.5 for testosterone, respectively.

2.6. Hormone assays

Faecal progestogen metabolites levels were measured using a progesterone monoclonal antibody (Quidel clone No. CL 425, Dr. Coralie Munro, University of California, Davis) diluted to 1:6000, horseradish peroxidise (HRP) conjugated progesterone 1:100,000 (C. Munro, University of California, Davis) and progesterone standards (200–0.39 pg/well). This antibody cross reacts with progesterone (100%), and a variety of reduced pregnane metabolites as determined by Graham et al. (2001).

Faecal glucocorticoid metabolites were measured using cortisol polyclonal antibody (R4866) diluted to 1:9000, HRP-conjugated cortisol 1:250,000 (C. Munro, University of California, Davis) and cortisol standards (1000–1.95 pg/well). This cortisol assay was successfully validated for other animals to provide reliable quantitative information regarding glucocorticoid output (Young et al., 2004, 2001) and found to cross-react with cortisol 100%, prednisolone 9.9%, prednisone 6.3% cortisone 5% and <1% with corticosterone, desoxycorticosterone, 21 desoxycortisone, testosterone, androstenedione, androsterone and 11-desoxycortisol (Young et al., 2004). The significant increase of faecal glucocorticoid levels

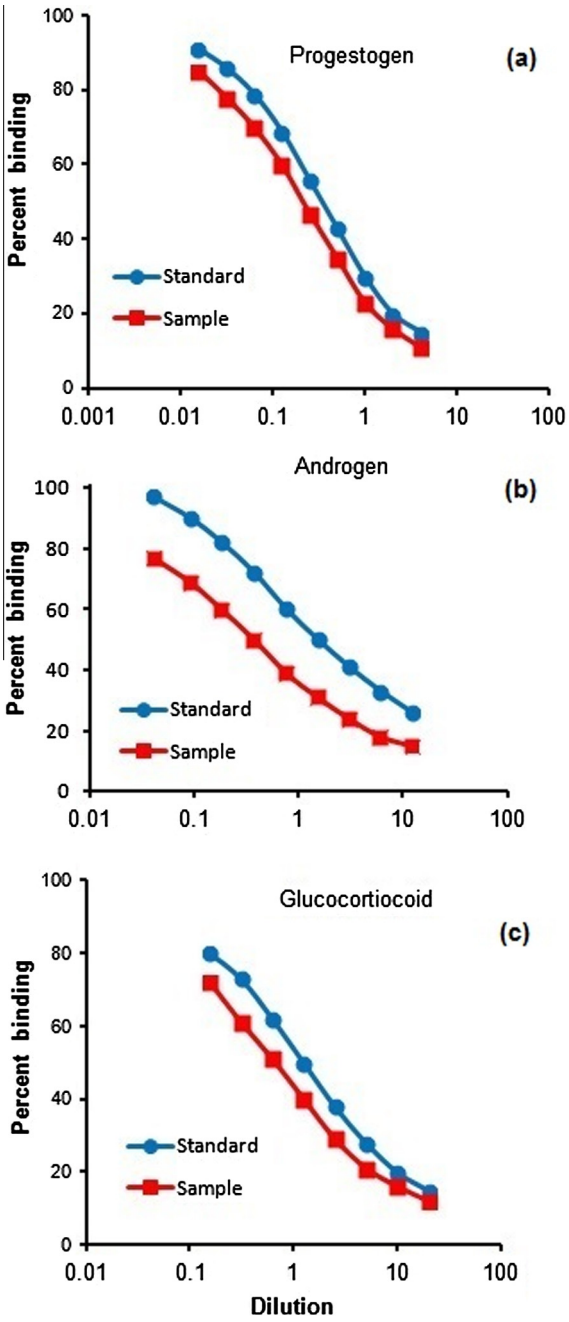


Fig. 2. Parallelism between serial dilutions of faecal extract of elephant (squares) and standards (circles) of progestogen (a), androgen (b) and glucocorticoid (c).

following public processions and temporary injury (Fig. 3c and a) could be considered as biological validation regarding the reliability of the assay used (Young et al., 2004; Ganswindt et al., 2010b).

Faecal androgen metabolites were measured using testosterone polyclonal antibody (R156/7) diluted to 1:10,000, HRP-conjugated testosterone 1:200,000 (C. Munro, University of California, Davis) and testosterone standards (600–1.17 pg/well).The testosterone antibody cross-reacts with dihydrotestosterone 57.4%, <0.3% with androstenedione and <0.1% with androsterone, dihydroepiandrosterone, β-estradiol and progesterone (Dloniak et al., 2004; Thongtip et al., 2008). The elevated faecal androgen levels following musth signs in all males (Fig. 4) could be considered as biological validation regarding the reliability of the assay used (Ganswindt et al., 2010a; Brown et al., 2007; Rasmussen et al., 1984).

2.7. EIA procedure and validation

The above enzyme immnuoassays were performed as described previously (Young et al., 2004; Munro and Lasley, 1988).

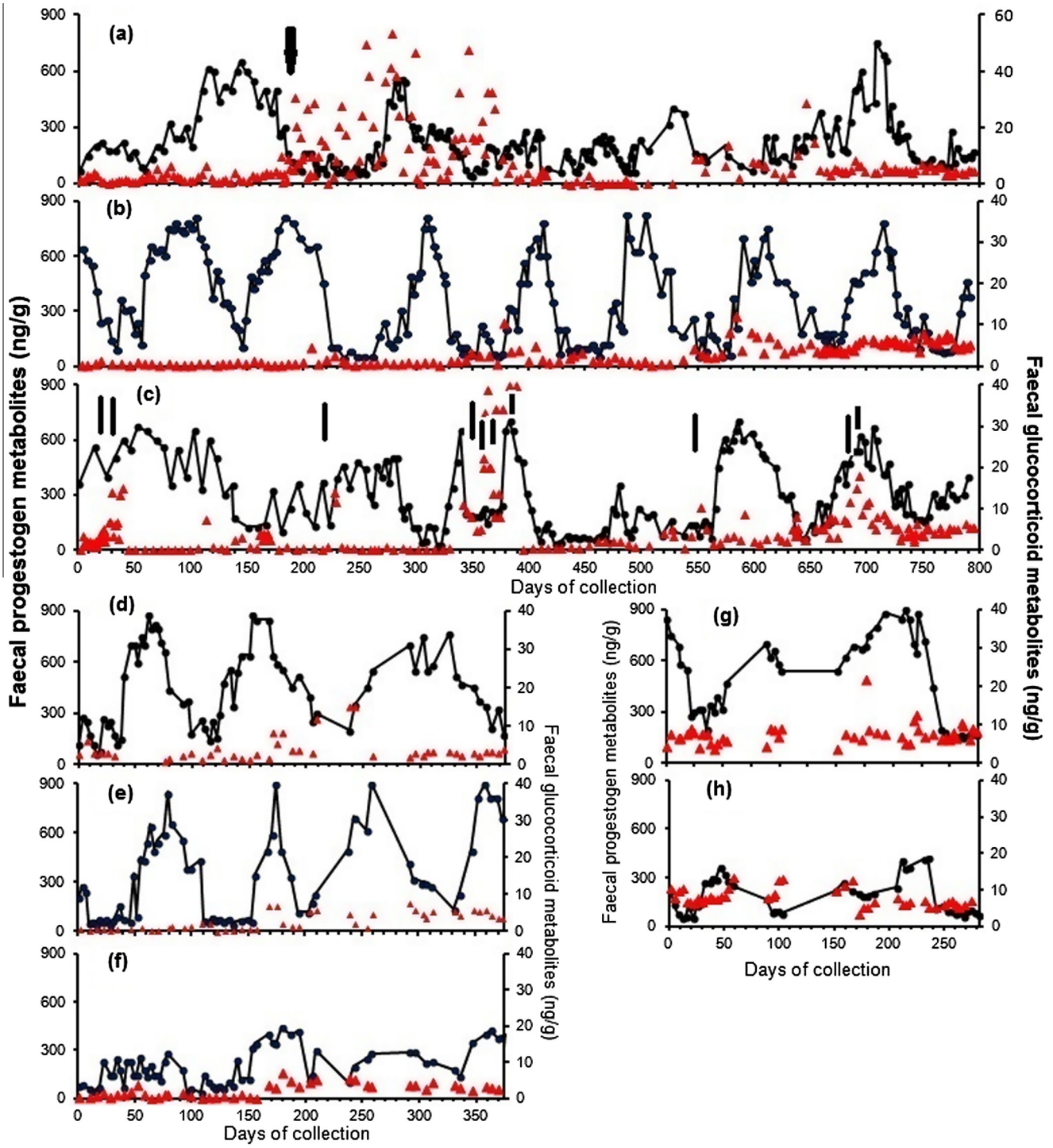


Fig. 3. Faecal progestogen (filled circle) and glucocorticoid metabolite concentrations (filled triangle – *Y1* axis) in eight females: (a) Asha, (b) Jamuna, (c) Vanaja (Hyderabad zoo), (d) Airavati, (e) Gajalakshmi, (f) Padmavati (Mysore zoo), (g) Rani and (h) Padmavathi (Tirupati zoo). The arrow pointing downwards indicates physical injury to the animal. Vertical bars indicate when the animal was taken out to participate in a public procession.

Respective antibodies were diluted in coating buffer (0.05 M sodium bicarbonate buffer, pH 9.6) to optimum dilution and coated (50 μl/well) onto 96-well plate (Nunc-Immuno maxisorp, Fisher scientific, USA), incubated overnight at 4 °C and washed four times with washing buffer (0.15 M NaCl and 0.05% Tween 20, Sigma, India). Steroid standards or faecal extracts (50 μl) diluted in assay buffer (0.1 M PBS, pH 7, containing 0.1% BSA) were added to the wells followed by 50 μl of conjugated HRPs steroids. After 2 h of incubation at room temperature, the plate was washed and 50 μl of TMB/H_2O_2 (Genei, Bangalore) substrate was added and kept in the dark for 10–15 min. The reaction was stopped by addition of 50 μl of 1 N HCl and absorbance read at 450 nm in the ELISA reader (Thermo Multiskan Spectrum Plate Reader, version 2.4.2, Thermo Scientific, Finland).

All EIAs were validated by demonstrating parallelism between the serial dilution of pooled faecal extracts (endogenous antigen) and respective standards (exogenous antigen) curves (Fig. 2a–c). Recovery of known amount of unlabelled steroids were 90.2 ± 3.3%, 88.37 ± 2.5 and 84.65 ± 4.5 for progesterone, testosterone and cortisol respectively in faecal extracts analysed by EIAs. The correlation (r^2) and slope (m) values for the recovery of exogenous progesterone, testosterone and cortisol were $r^2 = 0.99$, $m = 0.91$; $r^2 = 0.99$, $m = 0.98$; $r^2 = 0.99$, $m = 0.94$, respectively. Assay sensitivity was calculated at 90% binding. The assay sensitivities were found to be 0.39, 1.95 and 1.17 pg/well for progesterone, cortisol and testosterone EIAs, respectively. The intra and inter-assay coefficient of variation (CV) for progesterone, testosterone and cortisol were 3.40% and 7.82% ($n = 10$ plates), 6.84% and 8.55% ($n = 10$ plates) 2.98% and 5.30% ($n = 10$ plates), respectively.

2.8. Statistical analysis

Hormone values and oestrous cycle data are presented as mean ± SEM. For each male and female, peak and baseline concentrations were determined by using an iterative process as described by Brown et al. (1999). High values were removed if they exceeded mean plus 2 standard deviation (SD). The highest concentration in a group of elevated samples was considered as a peak and the remaining were considered as baseline values. Luteal phase was defined as the first day of increased faecal progestogen until it returned to the baseline value (concentrations were elevated for 5–7 consecutive days). Cycling was considered as based on the faecal progestogen and calculated as number of days from first peak until the next peak rise (Brown et al., 1999). Non-cycling elephants were categorised based on lack of clear faecal progestogen cycling profile for more than six months. Mann–Whitney U test (M–W test) was used for testing differences in faecal glucocorticoid and androgen metabolite levels between musth and non-musth animals, and between the two facilities. Kruskal–Wallis test (K–W test) was used for comparing faecal metabolites concentrations among different day storage condition and variation in body condition of individuals in three facilities. Spearman rank correlation coefficient (r_s) was used to examine association between two variables. We used a mixed effects multiple linear regression

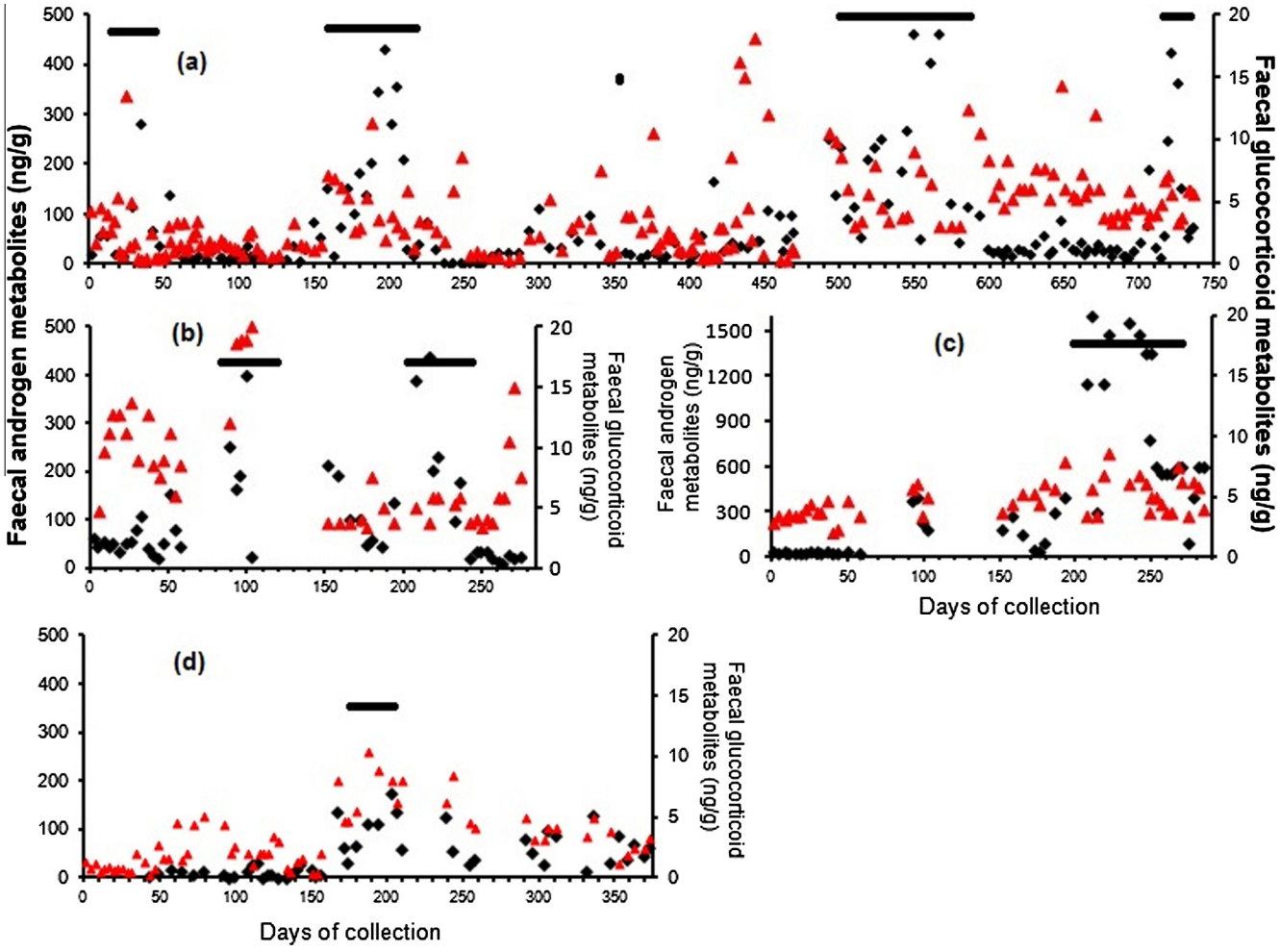


Fig. 4. Faecal androgen metabolite concentrations (filled square) and cortisol (filled triangle – *Y1* axis) concentrations in four male elephants: (a) Vijay (Hyderabad zoo), (b) Vinayaka (Tirupati zoo), (c) Venkanna (Tirupati zoo) and (d) Rama (Mysore zoo). The horizontal bars indicate observation of musth signs.

model to test differences in faecal glucocorticoid metabolites concentration among the facilities with reference to age and sex of individual elephants. All statistical analyses were carried out using SPSS 17.0.

3. Results

3.1. Elephant's body condition rating

The body condition ratings of the 12 elephants ranged from 4 to 9 and individual's body condition did not change during the study period. The body condition rating of Vijay, a male elephant from Hyderabad zoo was the lowest 4 followed by two females in the same zoo which were rated 6. The remaining 9 elephants were rated between 7 and 9 (Table 1). Two females, one each from Hyderabad and Mysore zoos, aged 39 and 33 years, respectively were rated highest as 9. Overall, no significant variation on the body condition was observed among the zoos (K–W $X^2 = 4.5$, $P = 0.11$) however Mysore zoo animals had better average body condition ratings (8.0) followed by Tirupati (7.75) and Hyderabad zoos (6.25). Furthermore, no significant correlation was observed between body condition and faecal glucocorticoid metabolites concentrations and age of the animals ($r_s = -0.009$; $P = 0.977$ and $r_s = -0.15$, $P = 0.64$, respectively). In males, a weak positive but non-significant correlation was observed between body condition and faecal androgen metabolite concentration ($r_s = 0.74$; $P = 0.26$).

3.2. Assessment of ovarian cyclicity using progestogen metabolites concentration

Maximum duration of monitoring was carried out in Hyderabad Zoo located in the same city as the laboratory where the analysis was carried out. For logistic reasons it was not possible to extend the duration of monitoring in Mysore and Tirupati zoos located 720 and 570 km away from the laboratory. Based on the faecal progestogen levels it was deduced that the average length of an estrous cycle was 16 weeks (range 14–17 weeks) (Fig. 3) with a follicular phase of 6 weeks (range 5–7 weeks) and luteal phases of 10 weeks (range 9–12 weeks), respectively. Based on these criteria it appeared that all females, except Padmavathi (Fig. 3f) showed cycling. However, Asha (Fig. 3a), Vanaja (Fig. 3c) and Gajalakhsmi (Fig. 3e) showed either long or short luteal phases during the study period. The mean faecal progestogen values for cycling females during the follicular and luteal phases were 98.54 ± 8.02 ng/g ($n = 120$, 16 phases across four females) and 619.45 ± 22.68 ng/g ($n = 122$, 16 phases four females), respectively.

3.3. Musth behaviour of elephants

All the males showed visible signs of musth at least once (maximum four times as in Vijay, aged 29 years, Hyderabad zoo, Fig. 4a), during the study period. The duration of musth varied from a minimum period of 15 days (Rama, 18 years, Mysore zoo, Fig. 4d) to a maximum period of 105 days (Vijay, 29 years, Hyderabad zoo, Fig. 4a). The duration of musth was not animal specific. For instance in Vijay, which exhibited signs of musth four times, the duration of its musth-period varies from 21 to 105 days (Fig. 4a).

3.4. Faecal androgen metabolite levels in male elephants

During non-musth individual mean faecal androgen metabolite levels ranged from 13.04 ± 1.25 ng to 37.38 ± 2.02 ng/g. While in musth they ranged between 253.90 ± 28.73 and 737.63 ± 89.54 ng/g (Fig. 4a and c). On average, elephants in musth showed three to four times increase in faecal androgen metabolites concentrations compared to individual basal levels, and remained elevated during the period of musth (Fig. 4a–d).

3.5. Faecal glucocorticoid metabolites in males

Individual mean basal faecal glucocorticoid levels ranged from 3.70 ± 0.28 to 5.59 ± 0.35 ng/g and increased prior to 2–3 weeks and during musth in three males Vijay, Venkanna and Rama (Fig. 4a, c and d). The fourth male (Vinayaka) did not show any difference in faecal glucocorticoid concentrations between musth and non-musth. All males, except Vinayaka ($r_s = -0.09$, $P = 0.54$, $n = 44$) showed significant positive correlation between faecal glucocorticoid and androgen concentrations (Vijay – $r_s = 0.29$, $P = 0.005$, $n = 96$; Rama – $r_s = 0.65$, $P = 0.0001$, $n = 51$; Venkanna – $r_s = 0.65$, $P = 0.0001$, $n = 47$).

3.6. Faecal glucocorticoid metabolites in females and during public processions/ festivals

Overall, individual mean basal faecal glucocorticoid metabolite concentrations in females ranged between 3.38 ± 0.27 ng/g and 9.31 ± 1.61 ng/g (Fig. 3a–h). It was also observed that Vanaja, the female from Hyderabad zoo, which was frequently used for public processions in Hyderabad city, exhibited very high faecal glucocorticoid concentrations (2–10 folds increase from the individual basal level) following or during such events (Fig. 3c).

3.7. Faecal glucocorticoid metabolite concentrations in relation to age and facility

Individual mean baseline faecal glucocorticoid metabolite concentrations ranged from 2.40 ± 0.33 ng/g to 9.4 ± 0.72 ng/g and the lowest was observed in the oldest female (58 years) and the highest in one of the younger females (12 years). Individual mean baseline faecal glucocorticoid metabolite concentrations did significantly differ for facility ($F_{1,188} = 49.53$, $P = 0.001$) and body condition ($F_{1,188} = 10.74$, $P = 0.001$) but did not differ for age ($F_{1,188} = 0.64$, $P = 0.42$) and sex ($F_{1,188} = 1.48$, $P = 0.22$). Overall, the mean baseline faecal glucocorticoid concentrations were significantly higher (7.60 ± 0.85 ng/g) in Tirupati Zoo animals compared to Hyderabad (4.67 ± 0.21 ng/g; M–W $U = 426$, $P = 0.001$) and Mysore zoo animals (3.42 ± 0.27 ng/g; M–W $U = 219$, $P = 0.001$).

4. Discussion

The present study reports on non-invasive reproductive and stress monitoring using about 1700 dung samples from 12 captive Asian elephants in 3 Indian zoos. Of the eight females, seven exhibited luteal activity, which includes five regular and two irregular cycling, and one did not show any luteal activity during the study period. The non-cycling female, Padmavathi (58 years), is oldest among the females and may have entered the post-reproductive phase. It may be noted that Padmavathi, is a proven fertile animal, which was reproductively active, and gave birth twice at the ages of 20 and 51 years.

The length of the estrous cycle in the three regular cycling elephants with a mean of 16 weeks is consistent with published literature (Brown et al., 2004; Ghosal et al., 2011). Three other elephants (Asha, Vanaja and Gajalakshmi) showed either lengthy or shorter cycles though they were in the reproductive age (33–42 years). The longer estrous period might be due to difference in environmental conditions (Thitaram et al., 2008), age and stress or due to physical injury. Glaeser et al. (2012) reported that the Asian elephants exhibited greater cycle variability (11–22 weeks)

among the individuals, which is different from previous reports (Brown, 2000; Thitaram et al., 2008). A similar observation was also made in a monitored Bornean elephant (Glaeser et al., 2012). One of the females in Hyderabad zoo, Asha, was stressed during the period of injury as evidenced from the elevated faecal glucocorticoid. A similar elevation faecal glucocorticoid metabolites levels was found in African male elephants due to foot injuries even though different EIAs were used (Ganswindt et al., 2010b). The other female in Hyderabad zoo, Vanaja (aged 42), was also in reproductive age, but showed longer, shorter, or disturbed luteal and follicular phases during the study period. This animal was used frequently (11 occasions) for public processions and festivals during the study period. At each occasion, it was subjected to presumed prolonged periods of stress since it was made to walk on hard tar roads between 8 am and 4 pm through the crowded roads and subjected to high decibel loudspeaker noise. That the animal was stressed is obvious from the fact that she had shown 2–10 folds increase in faecal glucocorticoid that could affect normal cycling. The longer and shorter luteal/follicular phases in the injured elephant (Asha) and the elephant (Vanaja) subjected to public procession/festivals could be attributed to the elevated stress level during the study period.

The other reasons for longer and irregular oestrus cycle in Hyderabad zoo elephants, might be due to lack of social interaction among the individuals and poor husbandry conditions leading to stress (Bradshaw et al., 2005). These elephants were chained and kept separately most of the time and were allowed very little time to interact with each other unlike the elephants at Mysore zoo, which were allowed to interact with other individuals between 8.00 am and 4.00 pm. Further, most of the females in Mysore zoological park were related to each other (e.g., Padmavathi and Airavati were mother and daughter) and their relatedness was very high compared to elephants at Hyderabad zoo. Studies have indicated that high group relatedness and social interaction could also be potential factors influencing positively the normal cycling of elephants (Gobush et al., 2008). This indeed may be true since the individual mean baseline faecal glucocorticoid metabolite concentration was lowest (3.13 ng/g) among Mysore zoo animals compared to others (Hyderabad 5.17 ng/g; Tirupati 7.17 ng/g). However, a more prolonged study on behavioural interaction might further improve our understanding about the influence of this factor on reproductive cycle.

The body condition of elephants did not show any significant correlation with any of the faecal hormones measured in the present study. However, in African elephants it was observed that the glucocorticoid concentrations were increased with declined body conditions (Foley et al., 2001). Overall, the body condition did not vary significantly with reference to age or facility and were rated mostly between 6 and 9. Thus, most of them were reproductively active.

All monitored males showed musth at least once during the study period, and the length of musth period varied from 15 days as in Rama (18 years) and 100 days as in Vijay (age 29 years) thus confirming the earlier studies of Prasad et al. (2000). Further two of the males (Vijay, Hyderabad and Vinayaka, Tirupati) showed musth twice a year within a gap of few months as in one of 29 males monitored in Kerala state of India for period of 10 years (Prasad et al., 2000). Ganswindt et al. (2003) indicated that the length of a musth period might depend on environmental conditions, access to females, age of the individual, etc. Since in the present study access to resources and age of males are similar, it is predicted that environmental conditions and access to females could be the major factors influencing the length of musth in our study animals.

In the present study, faecal androgen concentrations were higher during and prior to musth periods thus confirming earlier observations in both African (Ganswindt et al., 2005b; Rasmussen et al., 1996) and Asian elephants (Brown et al., 2007; Rasmussen and Perrin, 1999). Furthermore, three out of four males were showed significant positive correlations between faecal androgen and glucocorticoid metabolite concentrations during musth in our study animals. However, a potential co-measurement of faecal androgen metabolites with the glucocorticoid antibody (R4866) and vice versa of faecal glucocorticoid metabolites with antibodies of androgen (R156/7) cannot be excluded at this stage. Although the exact dynamics of glucocorticoid and androgen are not clearly understood, one possible hypothesis to explain our findings could be that during musth, the potentially more aggressive bulls are kept chained constantly which might result in an elevation of respective faecal glucocorticoid levels. Apart from that, musth was usually accompanied by significant weight loss because of reluctance of animals to consume food. Normally an elephant spends 90% of the time feeding but musth elephants spend less time feeding and this could be stressful resulting in elevated glucocorticoid. Ganswindt et al. (2003) did not find any significant rise in glucocorticoid during musth in African elephants. Recently, a similar observation was made on free-ranging Asian elephants (Ghosal et al., 2013). The observed difference in glucocorticoid elevation in Asian elephants and lack of it in African elephants might be due to the differences in management practices and may depend on individual animal's physiology.

5. Conclusion

This study once again highlights the importance of assessing ovarian cyclicity for an effective breeding program. Faecal progestogen was observed in all females and variation in cycling was observed. All the males exhibited signs of musth that coincided with peak concentration of faecal androgen metabolites. While relative changes in progestogens may be a critical factor in characterizing ovarian function in females, absolute concentrations of androgens seems more important for identifying musth in males. Monitoring faecal glucocorticoid metabolite levels may be a useful tool in evaluating stress levels in males and females. Management recommendation include periodic health care screening, keeping males and females together in a large enclosure during daytime and not allowing reproductively active females for public procession and festivals.

Acknowledgments

This study was funded by the Department of Science and Technology and Central Zoo Authority of India, Government of India. We thank Curators of Nehru zoological park, Hyderabad, Sri Venkateswara zoological park Tirupati and Sri Chamarajendra zoological gardens, Mysore for granting permission for sample collection.

References

Altmann, J., 1974. Observational study of behavior: sampling methods. Behaviour 49, 227–256.
Bradshaw, G.A., Schore, A.N., Brown, J.L., Poole, J.H., Moss, C.J., 2005. Elephant breakdown. Nature 433, 807.
Brown, J.L., 2000. Reproductive endocrine monitoring of elephants: an essential tool for assisting captive management. Zoo Biol. 19, 347–367.
Brown, J.L., Schmitt, D.L., Bellem, A., Graham, L.H., Lehnhardt, J., 1999. Hormone secretion in the Asian elephant (*Elephas maximus*): characterization of ovulatory and anovulatory luteinizing hormone surges. Biol. Reprod. 61, 1294–1299.
Brown, J.L., Walker, S.L., Moeller, T., 2004. Comparative endocrinology of cycling and non-cycling Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants. Gen. Comp. Endocrinol. 136, 360–370.
Brown, J.L., Somerville, M., Riddle, H.S., Keele, M., Duer, C.K., Freeman, E.W., 2007. Comparative endocrinology of testicular, adrenal and thyroid function in captive Asian and African elephant bulls. Gen. Comp. Endocrinol. 151, 153–162.

Brown, J.L., Kersey, D.C., Freeman, E.W., Wagener, T., 2009. Assessment of diurnal urinary cortisol excretion in Asian and African elephants using different endocrine methods. Zoo Biol. 29, 274–283.
Dloniak, S.M., French, J.A., Place, N.J., Weldele, M.L., Glickman, S.E., Holekamp, K.E., 2004. Non-invasive monitoring of faecal androgens in spotted hyenas (*Crocuta crocuta*). Gen. Comp. Endocrinol. 135, 51–61.
Foley, C.A.H., Papageorge, S., Wasser, S.K., 2001. Noninvasive stress and reproductive measures of social and ecological pressures in free-ranging African elephants. Conserv. Biol. 15, 1134–1142.
Ganswindt, A., Palme, R., Heistermann, M., Borragan, S., Hodges, J.K., 2003. Non-invasive assessment of adrenocortical function in the male African elephant (*Loxodonta africana*) and its relation to musth. Gen. Comp. Endocrinol. 134, 156–166.
Ganswindt, A., Heistermann, M., Hodges, J.K., 2005a. Physical, physiological and behavioural correlates of musth in captive African elephants (*Loxodonta africana*). Physiol. Biochem. Zool. 78, 505–514.
Ganswindt, A., Rasmussen, H.B., Heistermann, M., Hodges, J.K., 2005b. The sexually active states of free-ranging male African elephants (*Loxodonta africana*): defining musth and non-musth using endocrinology, physical signals, and behaviour. Horm. Behav. 47, 83–91.
Ganswindt, A., Münscher, S., Henley, M., Henley, S., Heistermann, M., Palme, R., Thompson, P., Bertschinger, H., 2010a. Endocrine correlates of musth and the impact of ecological and social factors in free-ranging African elephants (*Loxodonta africana*). Horm. Behav. 57, 506–514.
Ganswindt, A., Münscher, S., Henley, M., Palme, R., Thompson, P., Bertschinger, H., 2010b. Concentrations of faecal glucocorticoid metabolites in physically injured free-ranging African elephants (*Loxodonta Africana*). Wildl. Biol. 16, 323–332.
Ghosal, R., Kalaivanan, N., Sukumar, R., Seshagiri, P.B., 2011. Assessment of estrus cyclicity in the Asian elephant (*Elephas maximus*) by measurement of faecal progesterone metabolite 5a-P-3OH, using a non-invasive assay. Gen. Comp. Endocrinol. 175, 100–108.
Ghosal, R., Ganswindt, A., Seshagiri, P.B., Sukumar, R., 2013. Endocrine correlates of musth in free-ranging Asian elephants *(Elephas maximus)* determined by non-invasive faecal steroid hormone metabolite measurements. PLoS ONE 8, e84787.
Glaeser, S.S., Hunt, K.E., Martin, M.S., Finnegan, M., Brown, J.L., 2012. Investigation of individual and group variability in estrous cycle characteristics in female Asian elephants (*Elephas maximus*) at the Oregon Zoo. Theriogenology 78, 285–296.
Gobush, K.S., Mutayoba, B.M., Wasser, S.K., 2008. Long-term impacts of poaching on relatedness, stress physiology, and reproductive output of adult female African elephants. Conserv. Biol. 22, 1590–1599.
Graham, L., Schwarzenberger, F., Möstl, E., Galama, W., Savage, A., 2001. A versatile enzyme immunoassay for the determination of progestogens in feces and serum. Zoo Biol. 20, 227–236.
Hodges, J.K., 1998. Endocrinology of the ovarian cycle and pregnancy in the Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephant. Anim. Reprod. Sci. 53, 3–18.
Hunt, H.E., Wasser, S.K., 2003. Effects of long-term preservation methods on faecal glucocorticoid concentrations of grizzly bear and African elephant. Physiol. Biochem. Zool. 76, 918–928.
IUCN 2012. IUCN Red List of Threatened Species. Version 2012.2. http://www.iucnredlist.org/ downloaded on 25 April 2013.
Keay, J.M., Singh, J., Gaunt, M.C., Kaur, T., 2006. Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review. J. Zoo. Wildl. Med. 37, 234–244.
Lair, R.C., 1997. Gone astray: The care and management of the Asian elephant in domesticity, FAO/RAP Publication 1997/16, FAO Regional Office for Asia and the Pacific (RAP), Thailand.
Millspaugh, J.J., Washburn, B.E., 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. Gen. Comp. Endocrinol. 138, 189–199.
MoEF, 2004. Project elephant report. Ministry of Environment and Forests, Government of India. New Delhi.
Munro, C.J., Lasley, B.L., 1988. Non-radiometric methods for immunoassay of steroid hormones. In: Albertson, B.D., Haseltine, F.P. (Eds.), Non-radiometric assays: technology and application in polypeptide and steroid hormone detection. Alan R. Liss Inc., New York, pp. 289–329.
North American Regional Studbook, 2010. Asian Elephant Association of zoos and aquariums. Oregon USA, p. 235.
Poole, J.H., 1987. Rutting behavior in African elephants: the phenomenon of musth. Behaviour 102, 283–316.
Poole, J.H., Moss, C.J., 1981. Musth in the African elephant (*Loxodonta africana*). Nature 292, 830–831.
Prasad, A., Dinesh, M.T., Hareesh, P.S., Biju, S., Harikumar, S., Saseendran, P.C., 2000. Analysis of musth episodes in captive Asian elephants (*Elephas maximus*). Zoo's Print J. 15, 322–327.
Rasmussen, L.E.L., Perrin, T.E., 1999. Physiological correlates of musth: lipid metabolites and chemical composition of exudates. Physiol. Behav. 67, 539–549.
Rasmussen, L.E., Buss, I.O., Hess, D.L., Schmidt, M.J., 1984. Testosterone and dihydrotestosterone concentrations in elephant serum and temporal gland secretions. Biol. Reprod. 30, 352–362.
Rasmussen, L.E.L., Hall-Martin, A.J., Hess, D.L., 1996. Chemical profiles of male African elephants (*Loxodonta Africana*): physiological and ecological implications. J. Mammal. 77, 422–439.
Rees, P.A., 2003. Asian elephants in zoos face global extinction: should zoos accept the inevitable? Oryx 37, 20–22.
Slade-Cain, B.E., Rasmussen, L.E., Schulte, B.A., 2008. Estrous state influences on investigative, aggressive, and tail flicking behavior in captive female Asian elephants. Zoo. Biol. 27, 167–180.
Sukumar, R., 1989. The Asian elephant ecology and management. Cambridge University Press, Cambridge, UK, pp. 251.
Sukumar, R., 2003. Asian elephants in zoos – a response to Rees. Oryx 37, 23–24.
Thitaram, C., Brown, J.L., Pongsopawijit, P., Chansitthiwet, S., Wongkalasin, W., Daram, P., Roongsri, R., Kalmapijit, A., Mahasawangkul, S., Rojansthien, S., Colenbrander, B., Van der Weijden, G.C., Van Eerdenburg, F.J., 2008. Seasonal effects on the endocrine pattern of semi-captive female Asian elephants (*Elephas maximus*): timing of the anovulatory luteinizing hormone surge determines the length of the estrous cycle. Theriogenology 69, 237–244.
Thongtip, N., Saikhun, J., Mahsawangkul, S., Kornkaewrat, K., Pongsopavijitr, P., Songsasen, N., Pinyopummin, A., 2008. Potential factors affecting semen quality in the Asian elephant (*Elephas maximus*). Reprod. Biol. Endocrinol. 17, 6–9.
Umapathy, G., Kumar, V., Wasimuddin, Kabra, M., Shivaji, S., 2013. Detection of pregnancy and fertility status in big cats using an enzyme immunoassay based on 5α-pregnan-3α-ol-20-one. Gen. Comp. Endocrinol. 180, 33–38.
Vanitha, V., Thiyagesan, T., Baskaran, N., 2009. Socio-economic status of elephant keepers (*Mahouts*) and human–captive elephant conflict: a case study from the three management systems in Tamil Nadu, Southern India. Gajah 30, 8–12.
Wasser, S.K., Papageorge, S., Foley, C., Brown, J.L., 1996. Excretory fate of estradiol and progesterone in the African elephant (*Loxodonta africana*) and patterns of faecal steroid concentrations throughout the estrous cycle. Gen. Comp. Endocrinol. 102, 255–262.
Wemmer, C., Krishnamurthy, V., Shrestha, S., Hayek, L.A., Thant, M., Nanjappa, K.A., 2006. Assessment of body condition in Asian elephants (*Elephas maximus*). Zoo Biol. 25, 187–200.
Young, K.M., Brown, J.L., Goodrowe, K.L., 2001. Characterization of reproductive cycles and adrenal activity in the black-footed ferret (*Mustela nigripes*) by faecal hormone analysis. Zoo Biol. 20, 517–536.
Young, K.M., Walker, S.L., Lanthier, C., Waddell, W.T., Brown, J.L., 2004. Noninvasive monitoring of adrenocortical activity in carnivores by faecal glucocorticoid analyses. Gen. Comp. Endocrinol. 137, 148–165.

KERALA VETERINARY AND ANIMAL SCIENCES UNIVERSITY
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY – 680 651, THRISSUR, KERALA, INDIA
Ph: 0487 2370451, 2370344 (O), Telefax: 0487-2370388
E-mail: deanvetmty@kvasu.ac.in

DR. C. LATHA
DEAN

No: PA/Vet/29/2018 Dated 31.03.2018

To

Dr.Manilal Valliyat,
76, Ground Floor,
Defence Enclave, Vikas Marg,
New Delhi – 110 092.

Sir,

Sub: CVAS, Mannuthy – Results of screening of elephants for tuberculosis – results in original forwarding of – reg.

Ref; 1. Request from Animal Welfare Board of India dated 17.3.2018
2. Letter No. G3/0051/2016 dated 19.3.2018 of the Dean, CVAS, Mannuthy

As per the reference 1st cited, the screening test of the samples for Tuberculosis have been completed and the results in original is forwarded herewith in sealed envelope for favour of further necessary action.

Yours faithfully,

31/3/18

DEAN

Encl: as above.

COLLEGE OF VETERINARY AND ANIMAL SCIENCE MANNUTHY

DEAN
College of Veterinary & Animal Sciences
Kerala Veterinary & Animal Sciences University
Mannuthy, Thrissur-680 651

Results of Screening of elephants for tuberculosis using Chembio Kit

Sl No	Elephant ID No	Date of blood collection	Date of test	Diluent serial No.	RT Sl No.	Result
1	R87	17/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
2	R51	17/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
3	R16	17/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
4	R62	17/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
5	R63	17/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
6	R52	17/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
7	R53	17/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
8	R2	17/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
9	R34	17/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
10	R39	17/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
11	R30	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
12	R112	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
13	R13	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
14	R11	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
15	R55	18/01/2018	19/03/2018	B27083017	VTB063017/1	**Reactive**
16	R104	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
17	R72	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
18	R71	18/01/2018	19/03/2018	B27083017	VTB063017/1	**Reactive**
19	R1	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
20	R130	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
21	R58	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
22	R131	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
23	R125	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
24	R92	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
25	R27	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
26	R91	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
27	R20	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
28	R133	18/01/2018	19/03/2018	B27083017	VTB063017/1	**Reactive**
29	R14	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
30	R98	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
31	R107	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
32	R105	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
33	R56	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
34	R41	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
35	R116	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
36	R113	18/01/2018	19/03/2018	B27083017	VTB063017/1	**Reactive**
37	R81	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
38	R76	18/01/2018	19/03/2018	B27083017	VTB063017/1	**Reactive**
39	R126	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
40	R99	18/01/2018	19/03/2018	B27083017	VTB063017/1	**Reactive**
41	R102	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
42	R68	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
43	R100	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
44	R120	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
45	R110	18/01/2018	19/03/2018	B27083017	VTB063017/1	**Reactive**
46	R59	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
47	R24	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
48	R35	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
49	R17	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
50	R57	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive

Dr. BINU. K. MANI

Dr. Deepa. PM

51	R96	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
52	R109	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
53	R82	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
54	R83	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
55	R31	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
56	R23	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
57	R64	19/01/2018	19/03/2018	B27083017	VTB063017/1	**Reactive**
58	R49	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
59	R90	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
60	R18	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
61	R73	19/01/2018	19/03/2018	B27083017	VTB063017/1	**Reactive**
62	R75	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
63	R93	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
64	R33	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
65	R15	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
66	R115	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
67	R123	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
68	R128	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
69	R44	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
70	R7	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
71	R43	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
72	R122	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
73	R89	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
74	R9	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
75	R21	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
76	R117	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
77	R97	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
78	R80	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
79	R74	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
80	R95	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
81	R85	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
82	R111	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
83	R127	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
84	R134	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
85	R48	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
86	R77	20/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
87	R65	20/01/2018	19/03/2018	B27083017	VTB063017/1	**Reactive**
88	R94	20/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
89	Sonu	20/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
90	R25	20/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
91	R79	20/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive

Dr. Binu K Mani,
Assistant Professor,
Dept of Veterinary Microbiology,
CV& AS, Mannuthy,

Dr. Deepa P M, Assistant Professor
Department of Veterinary Epidemiology
and Preventive Medicine
CV&AS, Pookode

Countersigned:

Dr. K Vijayakumar, Professor & Head, Department of Veterinary Epidemiology and Preventive Medicine, CV&AS, Mannuthy

Dr. K. VIJAYAKUMAR
M.V.Sc., Ph.D.
Professor & Head
Dept. of Vety. Epi. & Preventive Medicine
Veterinary College, Mannuthy - 680 651

OPEN ACCESS Freely available online

PLOS ONE

Serodiagnosis of Tuberculosis in Asian Elephants (*Elephas maximus*) in Southern India: A Latent Class Analysis

Shalu Verma-Kumar[1,2☯], David Abraham[3☯], Nandini Dendukuri[4,5], Jacob Varghese Cheeran[6], Raman Sukumar[2], Kithiganahalli Narayanaswamy Balaji[1*☯]

1 Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, Karnataka, India, **2** Centre for Ecological Sciences, Indian Institute of Science, Bangalore, Karnataka, India, **3** Asian Nature Conservation Foundation, Bangalore, Karnataka, India, **4** Department of Medicine, McGill University, Montreal, Quebec, Canada, **5** Department of Epidemiology, Biostatistics and Occupational Health, McGill University, Montreal, Quebec, Canada, **6** Cheerans Lab (P) Limited, Thrissur, Kerala, India

Abstract

Background: *Mycobacterium tuberculosis*, a causative agent of chronic tuberculosis disease, is widespread among some animal species too. There is paucity of information on the distribution, prevalence and true disease status of tuberculosis in Asian elephants (*Elephas maximus*). The aim of this study was to estimate the sensitivity and specificity of serological tests to diagnose *M. tuberculosis* infection in captive elephants in southern India while simultaneously estimating sero-prevalence.

Methodology/Principal Findings: Health assessment of 600 elephants was carried out and their sera screened with a commercially available rapid serum test. Trunk wash culture of select rapid serum test positive animals yielded no animal positive for *M. tuberculosis* isolation. Under Indian field conditions where the true disease status is unknown, we used a latent class model to estimate the diagnostic characteristics of an existing (rapid serum test) and new (four in-house ELISA) tests. One hundred and seventy nine sera were randomly selected for screening in the five tests. Diagnostic sensitivities of the four ELISAs were 91.3–97.6% (95% Credible Interval (CI): 74.8–99.9) and diagnostic specificity were 89.6–98.5% (95% CI: 79.4–99.9) based on the model we assumed. We estimate that 53.6% (95% CI: 44.6–62.8) of the samples tested were free from infection with *M. tuberculosis* and 15.9% (97.5% CI: 9.8 - to 24.0) tested positive on all five tests.

Conclusions/Significance: Our results provide evidence for high prevalence of asymptomatic *M. tuberculosis* infection in Asian elephants in a captive Indian setting. Further validation of these tests would be important in formulating area-specific effective surveillance and control measures.

Citation: Verma-Kumar S, Abraham D, Dendukuri N, Cheeran JV, Sukumar R, et al. (2012) Serodiagnosis of Tuberculosis in Asian Elephants (*Elephas maximus*) in Southern India: A Latent Class Analysis. PLoS ONE 7(11): e49548. doi:10.1371/journal.pone.0049548

Editor: Jagadeesh Bayry, Institut National de la Santé et de la Recherche Médicale U 872, France

Received July 12, 2012; **Accepted** October 10, 2012; **Published** November 16, 2012

Copyright: © 2012 Verma-Kumar et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The study was funded by the Department of Science and Technology, Government of India by award of grant SR/WOS-A/LS-128/2009 to Dr. Shalu Verma-Kumar. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Jacob Varghese Cheeran is employed by Cheerans Lab (P) Limited. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials. Cheerans Lab (P) Limited had no role in developing the serological tests described in the current investigation.

* E-mail: balaji@mcbl.iisc.ernet.in

☯ These authors contributed equally to this work.

Introduction

Conservation medicine enables us to rethink the linkages between human, animal, and environmental health [1], [2]. As wildlife populations become more fragmented and less genetically diverse stochastic events leading to disease outbreaks could become more common. A case in hand is that of the Asian elephant (*Elephas maximus*) an ''Endangered'' flagship species and featuring on the '2010 IUCN Red List of Threatened Species' [3]. About 39,463-47,427 wild elephants are found in their 13 range countries in Asia [4]. Estimates of Asian elephant numbers in the wild in India are 26,000–28,000 of which 14,000 are found in southern India [5]. About 3467–3667 elephants are also held in captivity in India (http://envfor.nic.in/pe/PE%20Note.pdf) at forest camps, temples, zoological gardens and circuses, thus constituting a substantial population living in close proximity to humans.

Both humans and elephants are susceptible to infection primarily with *M. tuberculosis*. Importantly, TB is a serious zoonotic disease in elephants [6], [7], [8], [9], [10] and infects about 11-25% of tested captive elephant populations in USA, India and Nepal [11]. The transmission, pathobiology and immune correlates of TB are poorly understood in Asian elephants. There is paucity of information on the time intervals between exposure, seroconversion, and shedding of the bacilli as also latent versus active disease status. Equivalent to the culture of human sputum [12], [13], [14] trunk wash culture for isolation of *M. tuberculosis* remains the 'gold standard' of ante-mortem TB diagnostics in elephants [15]. In its absence, ante-mortem TB diagnosis presents a conundrum [16]. Clinical signs such as chronic weight loss, weakness, anorexia, exercise intolerance and abnormal discharge

from the trunk [17] are frequently absent or seen at the terminal stages. Intradermal tuberculin test [17], [18] and radiographic thoracic evaluation [17] are unsuccessful in elephants. The GenProbe Amplified *Mycobacterium tuberculosis* Direct Test (MTD; Gen-Probe, San Diego, CA, USA) has found limited use [19], while ELISA [17], [20], restriction fragment length polymorphism (RFLP) [6], [17], [18] and serological tests such as the dual path platform (VetTB test), multi-antigen print immunoassay (MAPIA) and the rapid serum test (RT, ElephantTB STAT-PAK) from Chembio Diagnostics (Medford, USA) have also been evaluated in elephants [21], [22], [23].

In this study, we report screening of elephants with RT and four in-house ELISAs using *M. tuberculosis* H37Rv antigens *EsxA*-6 kDa early secretory antigenic target (ESAT-6) (Rv3875); *EsxB*-10 kDa culture filtrate antigen (CFP10) (Rv3874); PE_PGRS17 (Rv0978c) and PE_PGRS11 (Rv0754). The ESAT-6 and CFP10 proteins function in inducing interferon gamma (IFNγ) from memory effector cells upon infection with pathogenic mycobacteria [24], [25]. The proline glutamic acid (PE) and proline-proline-glutamic acid (PPE) families of acidic, glycine-rich proteins are unique to the Mycobacteria [26] and many function as cell surface antigens. The PE_PGRS11 (Rv0754) is a hypoxia responsive gene that encodes a functional phosphoglycerate mutase [27] and PE_PGRS17 and PE_PGRS11 antigens induce maturation and activation of human dendritic cells [28].

Performance characteristics of a diagnostic test should ideally enable us to distinguish between infected and non-infected animals. Notably, estimation of DSe (the proportion of infected animals correctly identified by the diagnostic test) and DSp (the proportion of non-infected animals accurately identified by the diagnostic test) of any index test is generally derived by comparing it to a standardized and validated 'gold standard' with the assumed sensitivity and specificity of 100% [29], [30], [31]. However, the gold standard could suffer from serious deficiencies. For example, the ante-mortem trunk wash culture for *M. tuberculosis*/*M. bovis* in Asian elephants is reported to suffer from poor sensitivity [17], [22], logistical issues in sample collection and processing and slow turnaround time. In view of these observations, we have utilized Latent Class Analysis (LCA) of five imperfect serological tests to estimate and derive the probability of *M. tuberculosis* infection in elephants.

Results

Sampling in Elephants

About 600 captive Asian elephants were visited for health assessment in the three southern Indian states of Kerala, Karnataka and Tamil Nadu. The sampling included healthy individuals as well as animals with alternative diagnosis such as chronic arthritis, impaction, and other non-specific symptoms such as anemia and emaciation. Sera from 179 animals were randomly selected for this study. Trunk-wash culture for isolation of *M. tuberculosis* in select RT positive elephants was carried out with no elephant testing positive. Post-mortem examination of one RT positive elephant revealed lung nodules from which *M. tuberculosis* was cultured. Ante-mortem serum from this animal was used as positive control in standardizing the ELISAs and immunoblot assay.

Evaluation of Humoral Immunoreactivity

One hundred and seventy nine elephants were screened for differential B-cell responses using RT and recombinant *M. tuberculosis* H37Rv antigens in ELISA format. Only selected reference samples were tested by immunoblot analysis using the four recombinant antigens and the results were not included in the LCA model. The immunoblots (Figure 1) were not quantitative in nature. The RT readout gave either a positive or negative test result (binary outcome), while the ELISA results were continuous numerical outcome values (continuous outcome). We dichotomized each continuous test result using a Weibull mixture model that assumed the elephants were a mixture of two latent groups – those with the antigen and those without. The cut-off value was the point of intersection of the two Weibull distributions. The cut-off values were 0.2 for ESAT-6, 0.337 for CFP10 and 0.22 for both PE_PGRS11 and PE_PGRS17. Test value greater than each cut-off was deemed to be a positive test. Of the 179 elephants 33 tested positive in RT, 37 in ESAT-6 ELISA, 41 in CFP10 ELISA, 64 in PE_PGRS11 ELISA and 78 in PE_PGRS17 ELISA (Figure 1).

Analyzing Diagnostic Test Results using LCA

The LCA assumed in Figure 2 has sixteen latent classes. The sero-prevalence associated with each latent class is listed in Table 1. In the absence of any prior information, we chose to use a 'non-informative' prior distribution for the prevalence of *M. tuberculosis* infection, allowing for equal weight of all values from 0% to 100%. Using the posterior distribution, we report that 53.6% (97.5% CI: 44.6% to 62.8%) of the sera samples we tested did not carry any of the *M. tuberculosis* antibodies measured by the 5 tests. This estimate is higher than the 15% sero-prevalence reported by Abraham *et al.* (2008, Report submitted to Project Elephant, Ministry of Environment and Forests, Government of India) using RT in the same population of captive elephants. We report that the percentage of *M. tuberculosis* infected animals testing positive in all five tests is 15.9% (97.5% CI: 9.8%−24.0%). The DSe and DSp of each test with respect to the target antibody that it is designed to detect is listed in Table 2. Thus the PE_PGRS11 ELISA had the highest DSe of 97.6% (97.5% CI: 88.6%−99.8%) and DSp of 98.5% (97.5% CI: 93.6%−99.9%). Table 2 also lists the DSe and DSp of each test in detecting the presence of at least one antibody (Table 1 lists 15 latent classes that are positive for at least one antibody) as well as the latent antibodies it is not designed to detect. The main difference between our model and other latent class models is that we recognize that the different tests are measuring different latent variables. Thus, for example, we are able to comment not only on how well RT measures the antigens it is supposed to detect but also on how well it measures *M. tuberculosis* infection that is picked up by other antigens. Finally, the observed and predicted numbers of elephants for each combination of test results (Table 3) agree quite well suggesting that the model fits the data adequately.

Discussion

Latent class analysis is used in a scenario wherein the gold standard assessment of disease is unavailable and the true infection status unknown but the results of multiple imperfect tests are known [32], [33], [34]. This analysis is attractive because it does not arbitrarily treat one of the tests as a perfect gold-standard with 100% sensitivity and specificity. It allows intuitive construing of data for input and helps in understanding the uncertainties associated with the predicted prevalence estimates. A large number of reports using LCA in veterinary diagnostic tests have been published [35], [36], [37], [38], [39], [40]. This study uses LCA to estimate the diagnostic test characteristics of five serological tests and is the first report of this analysis used to study TB infection in elephants. This model recognizes that each

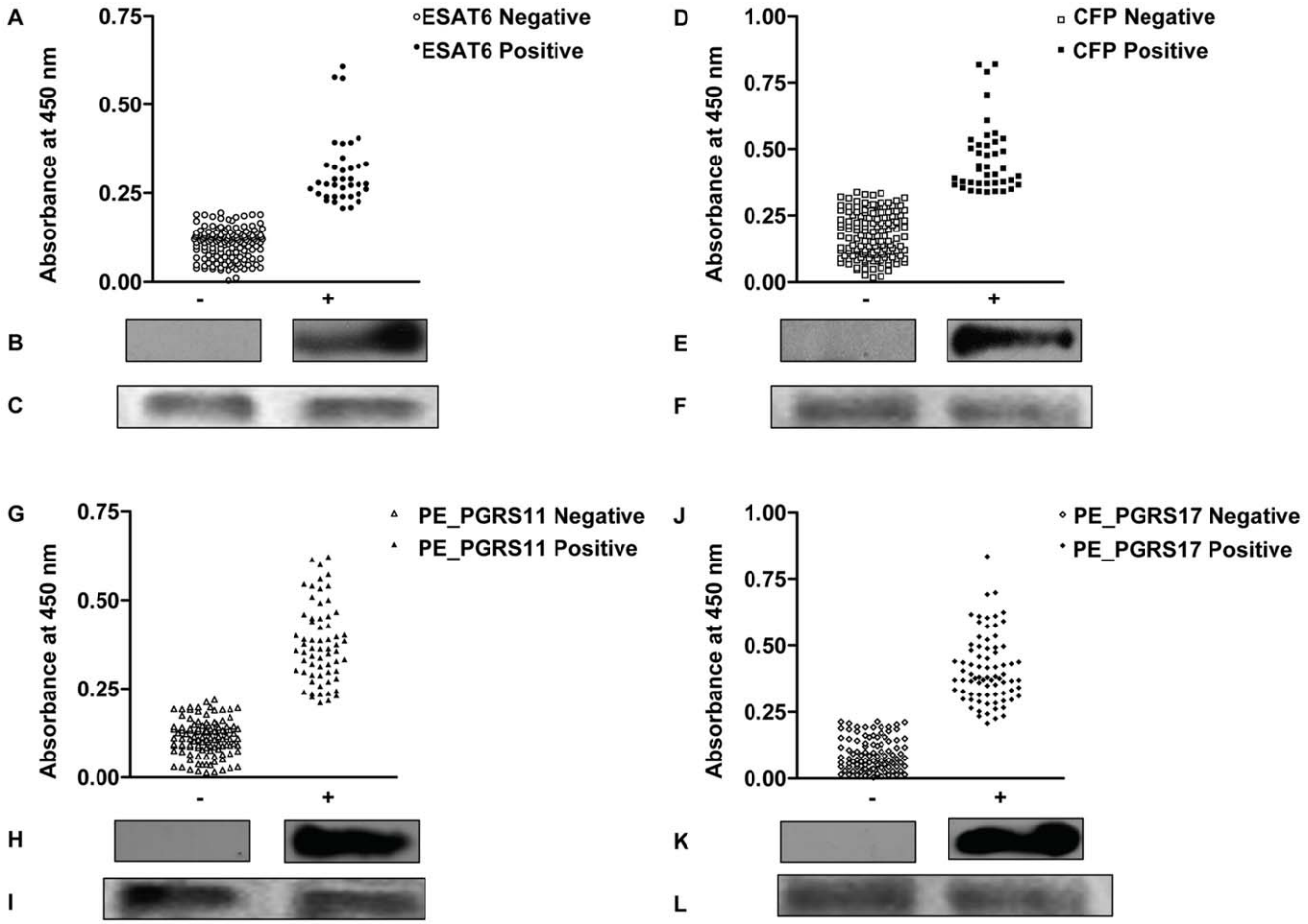


Figure 1. Differential humoral reactivity of four mycobacterial antigens with Asian elephant sera. For ELISA, elephant sera (1:200) was allowed to react with ESAT-6 (1 μg/ml) (A), CFP10 (0.5 μg/ml) (D), PE_PGRS11 (0.25 μg/ml) (G) and PE_PGRS17 (0.25 μg/ml) (J). Scatter plots show the total number of animals testing seronegative and seropositive for each antigen. For immunoblotting, 10 μg of each transferred protein ESAT-6 (C), CFP10 (F), PE_PGRS11 (I) and PE_PGRS17 (L) was first stained with Ponceau to check for loading control. Next, individual lanes were cut out of the blot and probed with sera from reference negative and positive animals. B, E, H, K represent immunoblots for one representative negative and positive animal each. The westerns were not quantitative in nature.
doi:10.1371/journal.pone.0049548.g001

test is measuring a different target latent variable, which is in turn associated with the presence of *M. tuberculosis* infection.

Test validation in the absence of suitable reference samples is extremely challenging [30], [31]. Culture for clinical isolation of *M. tuberculosis* remains the 'gold standard' of diagnostics; however a positive culture result is more likely in animals with advanced stage of disease. Validation of DSe based on positive culture results may result in its overestimation under field conditions [38], [40] and DSe may also vary with severity of disease. Validation of DSp entails testing of individuals and herds free from infection (a condition unlikely in TB endemic countries) [41] and may not be constant across different populations [38], [40]. Variables such as methods and their operational characteristics, expertise of the diagnostician, variations in host-pathogen interactions and difference in true disease prevalence rates may contribute to changes in performance of tests. Thus, test performance characteristics would need careful re-evaluation when used in different settings.

In order to decide the cut-off for our new in-house ELISAs, we had the choice of using either the RT as an imperfect gold standard or a mixture model. Even though the RT and ELISA work on the same biological principle, we did not use the former approach as the RT does not contain any PE_PGRS antigens. The mixture model for continuous data has been used for tuberculin skin test induration data among other applications [42]. The LCA is based on the premise that the true disease status is a common latent (or unobserved) variable associated with several imperfect tests that measure the same disease [43]. For the LCA, we delineated 16 latent classes which are the different combinations of the antibodies detected in the study. It would be interesting to link the latent classes to other disease parameters such as mortality and clinical signs. The disadvantage of using the LCA model is that it dichotomizes test results and thus does not use all the information derived from continuous test results. The choice of model used is dictated by the type of data and whether the model assumptions are satisfied. The model we propose is complex and subject to our interpretation. However, it incorporates results from five serological assays independent of culture results, estimates the true seroprevalence within the sampled population and the true disease status of each animal sampled therein.

Only about 10% of the *M. tuberculosis* proteome generates human antibody responses, and this immunoproteome contains predominantly membrane-associated and secreted proteins [44]. Differences in antibody profiles seen in TB patients need to factor in host characteristics, bacillary burden and metabolic state and

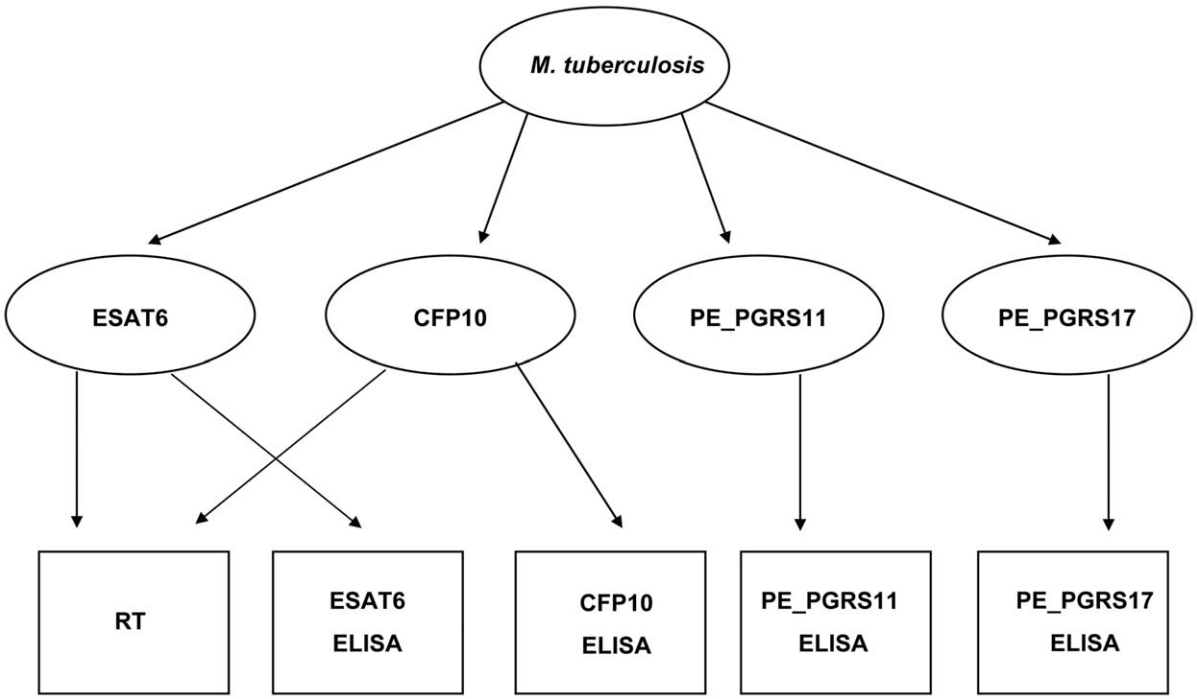


Figure 2. Schematic for the multiple latent variable model used in this study. The parameters to be estimated are depicted in oval shapes and the observed diagnostic test results are represented in rectangular shapes. While RT contains both the ESAT-6 and CFP10 antigens, the ELISAs are specific for one antigen each.
doi:10.1371/journal.pone.0049548.g002

protein expression by the infecting strain of *M. tuberculosis* [44], [45]. Additional complexity is introduced by the multiple clinical manifestations of disease in humans [46]. This host and pathogen derived heterogeneity has led to several reviews highlighting the shortcomings of TB immunodiagnostics [47], [48], [49], [50].

Circulating antibodies have been evaluated as biomarkers for TB since 1898 [51]. As compared to other members of the PE family (including PG_PGRS) or mycobacterial antigens, PE_PGR11 and PE_PGR17 have been shown to elicit stronger and differential antibody response in humans. Our laboratory has previously reported that PE_PGR11 and PE_PGR17 elicited antibodies in adult humans with active pulmonary infection, and in child patients with pulmonary or extrapulmonary TB [27], [28], [52]. Serology studies have demonstrated that antibodies reactive with a recombinant carboxyterminal fragment of the PE_PGRS protein from Rv1759c [53] or with the PGRS domain of Rv3367 [54] are present in human sera of patients infected with TB. The ESX protein family (for example, Rv3881c and Rv3784) are preferentially recognized antibody targets in active TB in humans [44]. The proteins CFP10 (Rv3784) and ESAT6 (Rv3785) have been evaluated in a number of veterinary serological assays [21], [22], [55], [56]. This approach is now giving way to whole proteome screening to identify TB associated proteins and the dynamics of antibody response they elicit during disease.

The past decade has seen decline of Asian elephant populations in most range countries with the exception of India and Sri Lanka [4]. Population depletion reduces the risk of host-specific infectious diseases except when the pathogen resides in reservoir hosts or when captivity results in increased infectious disease transmission [57]. Thus, conservation strategies that increase population density or cross-species contact such as in zoos, reserves and other captive conditions need careful evaluation in light of the risks of such infection. Cases of cross-species TB outbreaks in zoos are well documented [58]. The management of TB in Asian elephants in India remains inadequate owing to a number of issues including historical and cultural context of captivity of the species, legality of ownership (government owned vs. privately- owned) or the costs associated with the treatment. Albeit the United States Department of Agriculture has drafted clear guidelines [15] current need involves a creation of guidelines in regard to regulation of TB infections in elephants in India. Thus, our current study clearly proposes periodic verification/testing for TB in captive elephants as well as in-contact personnel.

Factors governing the initiation and expansion of ensuing immunity to wide ranging infections in elephants are not well understood. Further, detailed analysis of various effector roles played by the key components of immune systems such as T cells, B cells, macrophages or dendritic cells, complement and cytokines requires extensive investigation. For example, presence of five subclasses of IgG has been demonstrated in African elephant (*Loxodonta africana*) [59], [60]. Further, the genomic organization of the IgH, Igκ, and Igλ loci of the African elephant has been identified [61], [62]. Immunoreactivity analysis demonstrated the role for complement and antibodies during infections with African horse sickness [63], [64] Bluetongue [63] and Mycoplasmosis [65]. Interestingly, Alpha Napthyl Acetate Esterase activity was utilized as a T cell marker to demonstrate T lymphocyte distribution in peripheral blood [66] and presence of functional CD genes in the African elephant [67]. Not much tuberculosis disease stage-specific information is available in Asian elephants and vaccination with *M. bovis* BCG has not been evaluated in elephants. Lyaschenko *et al.* [21] reported that Multiantigen print immunoassay (MAPIA) and RT could pick up serum IgG to ESAT-6 and other proteins up to 3.5 years and 4.0 years respectively prior to culture of *M.*

Table 1. Defining the sixteen latent classes and calculating their sero-prevalence rates.

Class No.	Latent class definition	Seroprevalence	
		Median (95% CI)*	Mean
Class 1	Mtb**+, All antibodies+	15.9 (9.8, 24.0)	16.2
Class 2	Mtb+, ESAT6+, CFP10+, PE-PGRS11+	1.3 (0.1, 4.3)	1.5
Class 3	Mtb+, ESAT6+, CFP10+, PE-PGRS17+	0.4 (0.0, 2.1)	0.6
Class 4	Mtb+, ESAT6+, PE-PGRS11+, PE-PGRS17+	0.8 (0.0, 4.4)	1.2
Class 5	Mtb+, CFP10+, PE-PGRS11+, PE-PGRS17+	3.1 (0.2, 10.3)	3.7
Class 6	Mtb+, ESAT6+, CFP10+	0.8 (0.0, 3.2)	1.0
Class 7	Mtb+, ESAT6+, PE-PGRS11+	0.6 (0.0, 3.2)	0.9
Class 8	Mtb+, ESAT6+, PE-PGRS17+	0.5 (0.0, 2.3)	0.7
Class 9	Mtb+, CFP10+, PE-PGRS11+	0.5 (0.0, 2.6)	0.7
Class 10	Mtb+, CFP10+, PE-PGRS17+	0.4 (0.0, 2.3)	0.6
Class 11	Mtb+, PE-PGRS11+, PE-PGRS17+	11.9 (4.2, 18.5)	11.7
Class 12	Mtb+, ESAT6+	0.7 (0.0, 3.5)	1.0
Class 13	Mtb+, CFP10+	0.5 (0.0, 2.6)	0.7
Class 14	Mtb+, PE-PGRS11+	0.7 (0.0, 3.1)	0.9
Class 15	Mtb+, PE-PGRS17+	4.8 (0.2, 11.8)	5.1
Class 16	Mtb-, All antibodies-	53.6 (44.6, 62.8)	53.6

Latent class definition and the seroprevalence rate for each class thus defined in the LCA model for studying TB in Asian elephants in southern India. The latent classes are different combinations of the latent variables (i.e. true presence of the antibodies) which are present if *M. tuberculosis* infection is present. *Median estimate is at the 50% quantile while the 2.5% and 97.5% quantiles define a 95% credible interval (CI). ***M. tuberculosis* infection.

doi:10.1371/journal.pone.0049548.t001

tuberculosis from trunk washes. They reported that ESAT6 and CFP10 were the immunodominant antigens elicited upon infection of elephants with *M. tuberculosis*/*M. bovis* [21], [23]. Significantly, in addition to ESAT-6 and CFP-10, we report high DSe and DSp for the two PE_PGRS ELISAs based on a latent class model that recognizes that the different tests are designed to measure different antigens. The DSe and DSp for the ELISAs we developed for ESAT6 and CFP10 were comparable to the commercially available RT test. Importantly, we have attempted to address immunoreactive potential as well as serodiagnostic utility of selected antigens of *M. tuberculosis* H37Rv which would help in our understanding of the pathophysiological attributes of TB infection in elephants.

Serology remains an attractive first step in TB diagnosis in wildlife. It is simple, quick, affordable and does not require repeated handling of animals. Once elicited, the antibody response is sustained while the trunk wash culture may yield intermittent results in elephants. Identifying serological correlates of active TB in elephants and their use in antitubercular treatment monitoring [21], [23], [68] could be potentially useful tools in situations where it is important to keep costs of diagnosis low. We are currently evaluating cell immunity based assays for TB diagnosis in elephants. Further studies into TB transmission and surveillance using accurate, low cost and high throughput assays are also warranted. Such active disease surveillance in elephant range countries would help us to study the dynamic relationship between TB and elephant conservation.

Table 2. Sensitivity (DSe) and specificity (DSp) of the five serological tests used in the study.

Test	w.r.t.**	DSe	DSp
		Median (95% CI)*	Median (95% CI)*
RT	At least 1 antibody	48.6 (37.2, 61.0)	99.3 (96.7, 99.9)
	ESAT6	44.6 (33.9, 56.4)	95.2 (90.4, 98.2)
	CFP10	46.5 (36.0, 58.1)	93.0 (87.8, 96.6)
	PE_PGRS11	79.8 (66.7, 90.8)	98.5 (93.6, 99.9)
	PE_PGRS17	84.5 (75.2, 91.3)	89.6 (79.4, 98.4)
ESAT6	At least 1 antibody	88.8 (73.5, 97.5)	95.1 (90.5, 98.2)
	ESAT6	91.3 (74.8, 99.5)	95.2 (90.4, 98.2)
	CFP10	77.0 (61.9, 88.5)	88.9 (83.2, 93.6)
	PE_PGRS11	85.0 (71.7, 94.0)	75.9 (68.2, 82.3)
	PE_PGRS17	80.3 (66.7, 90.2)	65.7 (57.8, 73.1)
CFP10	At least 1 antibody	88.8 (73.5, 97.5)	95.3 (90.8, 98.2)
	ESAT6	75.2 (60.0, 87.1)	91.2 (85.7, 95.2)
	CFP10	92.3 (76.9, 99.5)	93.0 (87.8, 96.6)
	PE_PGRS11	85.2 (71.5, 94.0)	76.0 (68.5, 82.6)
	PE_PGRS17	81.5 (67.9, 91.4)	66.2 (58.3, 73.5)
PE_PGRS11	At least 1 antibody	50.1 (38.2, 62.1)	93.9 (88.8, 97.2)
	ESAT6	47.1 (35.8, 58.7)	91.5 (85.9, 95.4)
	CFP10	49.1 (38.0, 60.2)	89.4 (83.5, 93.7)
	PE_PGRS11	97.6 (88.6, 99.8)	98.5 (93.6, 99.9)
	PE_PGRS17	87.1 (77.6, 93.6)	81.4 (73.4, 88.1)
PE_PGRS17	At least 1 antibody	44.4 (32.5, 57.6)	91.6 (85.5, 96.0)
	ESAT6	42.2 (30.9, 54.8)	89.6 (83.0, 94.1)
	CFP10	44.8 (33.6, 57.1)	87.8 (81.5, 92.7)
	PE_PGRS11	82.7 (68.2, 94.3)	91.9 (85.3, 96.5)
	PE_PGRS17	97.2 (88.5, 99.9)	89.6 (79.4, 98.7)

The LCA model was used to calculate the DSe and DSp of each test w.r.t. the antibody it is designed to detect as also presence of at least one antibody. *The Median estimate refers to the 50% quantile while the 2.5% and 97.5% quantiles define a 95% credible interval (CI). ** With respect to.

doi:10.1371/journal.pone.0049548.t002

Materials and Methods

Study Population

In the three southern Indian states of Kerala, Karnataka and Tamil Nadu, there are an estimated 1,000 Asian elephants in captivity. These animals, mostly caught from the wild but also born in captivity, are maintained under different ownership and management regimens. A project for captive elephant health assessment was undertaken by Asian Nature Conservation Foundation (Permit No.8-1/2002-PE, Project Elephant, Ministry of Environment and Forests, Government of India). Apart from photographic documentation of body condition index, wounds and injuries with special reference to eyes and feet, routine haematology, serum biochemistry, urinalysis and dung analysis were performed for individual elephants. In an attempt to provide better healthcare to these elephants, the results of each elephant's health evaluation was then handed over to the veterinarian in-charge of the elephant. Following a convenience/opportunity sampling method, nearly 600 elephants were visited over a period of one year. A random sample of 179 serum samples collected from this heath survey was selected for this study.

Table 3. Number of elephants (observed and predicted according to the latent class model) with each test result.

Binary Input	2.50%	50%	97.50%	Mean	Observed
00000	69	82	92	81	84
00001	9	16	26	17	16
00010	0	3	8	3	1
00011	18	24	29	24	26
00100	1	6	13	6	8
00101	0	1	4	1	1
00110	0	0	2	0	1
00111	0	2	6	2	0
01000	0	4	11	4	4
01001	0	1	3	1	1
01010	0	0	2	0	0
01011	0	1	5	2	2
01100	0	0	2	0	0
01101	0	0	1	0	0
01110	0	0	2	0	0
01111	0	2	8	3	2
10000	0	0	4	1	0
10001	0	0	1	0	0
10010	0	0	1	0	0
10011	0	1	3	1	1
10100	0	0	2	0	0
10101	0	0	2	0	0
10110	0	0	2	1	0
10111	1	4	8	4	4
11000	0	0	2	0	0
11001	0	0	2	0	0
11010	0	1	3	1	1
11011	0	3	8	3	2
11100	0	1	2	1	1
11101	0	0	3	1	0
11110	0	2	5	2	2
11111	12	19	25	19	22

Evaluating the fit of the model by comparing the observed and expected number of elephants with different combinations of tests to see if the assumptions of the substantive model in Figure 2 were satisfied. 50% refers to the median estimate while the 2.5% and 97.5% quantiles define a 95% credible interval (CI).

doi:10.1371/journal.pone.0049548.t003

Materials

Unless otherwise specified all materials used in this study were purchased from Sigma-Aldrich, St. Louis, USA. Vacutainer needles (No. 301747), Vacutainer tubes (No. 367820), PANTA^{TM} Supplement, BACTEC^{TM} 12B Mycobacteria Culture Vials and BBL^{TM} Mycobactose Lowenstein-Jensen Medium were procured from BD (Franklin Lakes, NJ, USA). GammaBind G, Type 2 affinity matrix was bought from GE Healthcare Bio-Sciences (Uppsala, Sweden), Ni-nitrilotriacetic acid (Ni-NTA) columns from Qiagen (Valencia, CA, USA), Nunc-Immuno Plates (No 44204) from NUNC A/S (Roskilde, Denmark) and polyvinylidene difluoride membranes (PVDF) from Millipore (Bedford, MA, USA). ECL detection system was bought from Perkin-Elmer (MA, USA) and 3,3′,5,5′-tetramethylbenzidine (TMB) and horse radish peroxidase (HRP)-labeling kit from Bangalore Genei (Bangalore, Karnataka, India).

Collection of Serum and Screening with RT

Blood was collected by venipuncture of the middle auricular vein, allowed to clot at room temperature and serum separated within three hours of collection by centrifugation at 500 *g* for 10 minutes. Each serum was screened with RT and stored at −70°C until further testing. The RT (http://www.chembio.com/animaltest4.html) is a point-of-care lateral flow serological test licensed by the USDA in 2007. Greenwald *et al.* [22] reported a DSp of 95.2% (95% CI, 90.1 to 97.9) and DSe of 100% (95% CI, 84.0%−100%) for the RT.

Trunk-wash Culture for Isolation of *M. tuberculosis*

The procedure for trunk wash collection as described in the Guidelines for the Control of Tuberculosis in Elephants, 2008 [15] was modified [69]. Trunk wash specimens from select RT positive elephants were tested for *M. tuberculosis* culture [15]. Briefly, 0.5 ml of sample supplemented with Erythromysin (32 µg/ml) and PANTA^{TM} Supplement was inoculated into BACTEC^{TM} 12B vials and BBL^{TM} Mycobactose Lowenstein-Jensen Medium and grown at 37°C and 10% CO_2 for 8 weeks; this was followed by a niacin-nitrate reduction test for confirming *M. tuberculosis*. Ante-mortem serum collected from one elephant, which showed nodules in the lung tissue during post-mortem examination and from which *M. tuberculosis* was cultured on Lowenstein-Jensen medium, was used as positive control in the serological assays.

Expression and Purification of Recombinant ESAT6, CFP10, PE-PGRS17 AND PE-PGRS11 Proteins

M. tuberculosis H37Rv antigens *EsxA*-6 kDa early secretory antigenic target (ESAT-6) (Rv3875); *EsxB*-10 kDa culture filtrate antigen (CFP10) (Rv3874); PE_PGRS17 (Rv0978c) and PE_PGRS11 (Rv0754) were used as the major antigenic determinants in this study. Recombinant expression vectors for Rv3875, Rv3874, Rv0978c and Rv0754 antigens were obtained from Colorado State University, TB Vaccine Testing and Research Materials Contract (http://www.cvmbs.colostate.edu/mip/tb/recombinant.htm) and expressed as described previously [70]. Briefly, for the expression of His-tagged recombinant antigens, *Escherichia coli* BL21(DE3) cells carrying recombinant plasmids were induced with isopropyl-b-D-thiogalactopyranoside, and the proteins purified under native conditions (ESAT6 and CFP10) and denaturing conditions (PE_PGRS17 and PE_PGRS11) using Ni-NTA columns. In-gel digestions of proteins for matrix-assisted laser desorption/ionization mass spectrometry was carried out for identification.

Raising Rabbit Anti-Asian Elephant IgG-horse Radish Peroxidase (HRP)

Asian elephant IgG was separated from sera [71] and rabbit anti-Asian elephant IgG raised as per [59] and [60] with modifications. Briefly, New Zealand white rabbits were injected subcutaneously at multiple sites with 1 mg of purified Asian elephant IgG emulsified in equal volume of Freund's complete adjuvant followed by a second dose of 500 µg Asian elephant IgG emulsified in Freund's incomplete adjuvant. Antibody titres in sera were determined two weeks post final immunization by ELISA. The rabbit anti-Asian elephant IgG was coupled to HRP and its reactivity to elephant IgG was checked. All animal experiments were approved by the Institutional Ethics Committee for Animal

Experimentation and Institutional Biosafety Committee, Indian Institute of Science, Bangalore.

ELISA

Careful checker board titration for optimum protein concentration, elephant sera dilution, rabbit anti-Asian elephant IgG-HRP was carried out for each individual ELISA. All protein dilutions were made in 1X PBS (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na2HPO4; 1.47 mM KH2PO4, pH 7.4). 1X PBST (1X PBS with 0.05% tween-20) was used as wash buffer and 3% BSA in PBST as blocking buffer. Elephant sera, rabbit anti-Asian elephant IgG-HRP were each diluted in blocking buffer and 100 µl added per well. ESAT-6 (1 µg/ml), CFP10 (0.5 µg/ml), PE_PGRS17 and PE_PGRS11 (0.25 µg/ml each) were coated overnight (o/n) at 4°C into ELISA plates and then washed thrice. Blocking for 1 hour was followed by addition of elephant sera (1:200) and incubation o/n at 4°C. After washing, rabbit anti-Asian elephant IgG-HRP (1:3000) was added and the plate incubated o/n at 4°C. Tetramethylbenzidine was used as chromogenic substrate and the absorbance was read at 450 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Immunoblot Analysis

Ten µg of purified protein was subjected to 12% SDS-PAGE (Laemmeli) or 10% Tricine SDS-PAGE; transferred to PVDF and stained with Ponceau to check for loading control. The PVDF was cut into strips, blocked with 5% nonfat dried milk and each strip probed with individual elephant sera overnight at 4°C, probed with rabbit anti-Asian elephant IgG-HRP and the blot visualized with the ECL detection system.

Statistical Analysis

To adjust for the imperfect nature of the gold-standard reference, our test validation entailed i) determining an optimal cut-off for each ELISA using a mixture model for continuous data ii) using LCA to estimate the DSe and DSp of the five dichotomous tests. The first step was carried out using the *mixdist* library in the R software package [72] assuming that the observed continuous data on each test arises from a mixture of two Weibull distributions among the antibody positive and antibody negative elephants. The point of intersection of the two density functions was chosen as the optimal cut-off.

The LCA was carried out using the *lcmr* library in R software package [43]. This package uses a Bayesian approach to estimate the parameters of interest. We used non-informative prior distributions over all parameters so as to let the data dominate the analysis. The LCA model (Figure 2) assumed that each of the ELISA tests was measuring a different latent variable (i.e. true presence of the antibody) which was present if *M. tuberculosis* infection was present. Thus, this model adjusts for the correlation that may arise between tests within the groups of elephants that are *M. tuberculosis* infection positive or negative. The RT test was assumed to measure the presence of both ESAT-6 and CFP10 antibodies while each of the four ELISA was assumed to measure the presence of the corresponding antibody. The resulting model had 16 latent classes corresponding to different combinations of the antibodies (see supplementary document File S1). The fit of the model was evaluated by comparing the observed and expected number of elephants with different combinations of tests and a posterior predictive check for conditional dependence (Table 3). The predictive values of each test combination were examined to see if the assumptions of the substantive model in Figure 2 were satisfied.

Supporting Information

File S1 Algorithm for LCA model.
(DOC)

Acknowledgments

We gratefully acknowledge the support extended by Director, Project Elephant, Ministry of Environment and Forests, Government of India, and the state forest departments of Karnataka, Tamil Nadu and Kerala. We thank the TB Vaccine Testing and Research Materials Contract at Colorado State University for the gift of recombinant expression vectors used in this study. We acknowledge help by B.C. Chittiappa and other field veterinarians. We are grateful to Thomas Mathew, Kavita Iswaran, Karpagam Chelliah, Devram Sampat Ghorpade, Sahana Holla, Kushagra Bansal, Nisha Kapoor and Yeddula Narayana for their contributions to this study.

Author Contributions

Conceived and designed the experiments: SVK DA KNB RS. Performed the experiments: SVK DA. Analyzed the data: ND SVK. Contributed reagents/materials/analysis tools: RS JVC ND KNB. Wrote the paper: SVK ND DA RS KNB.

References

1. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, et al. (2008) Global trends in emerging infectious diseases. Nature 451: 990–993.
2. Aguirre AA, Gomez A (2009) Essential veterinary education in conservation medicine and ecosystem health: a global perspective. Rev Sci Tech 28: 597–603.
3. Choudhury A, Lahiri Choudhury DK, Desai A, Duckworth JW, Easa PS, et al. (2010) Elephas maximus. IUCN 2010. IUCN Red List of Threatened Species Version 20102, 2010.
4. Fernando P, J P (2011) Range-wide Status of Asian Elephants. Gajah 35: 15–29.
5. Bist SS (2006) Elephant conservation in India - an overview. Gajah 25: 27–37.
6. Michalak K, Austin C, Diesel S, Bacon MJ, Zimmerman P, et al. (1998) *Mycobacterium tuberculosis* infection as a zoonotic disease: transmission between humans and elephants. Emerg Infect Dis 4: 283–287.
7. Davis M (2001) *Mycobacterium tuberculosis* risk for elephant handlers and veterinarians. Appl Occup Environ Hyg 16: 350–353.
8. Montali RJ, Mikota SK, Cheng LI (2001) *Mycobacterium tuberculosis* in zoo and wildlife species. Rev Sci Tech 20: 291–303.
9. Oh P, Granich R, Scott J, Sun B, Joseph M, et al. (2002) Human exposure following *Mycobacterium tuberculosis* infection of multiple animal species in a Metropolitan Zoo. Emerg Infect Dis 8: 1290–1293.
10. Murphree R, Warkentin JV, Dunn JR, Schaffner W, Jones TF (2011) Elephant-to-human transmission of tuberculosis, 2009. Emerg Infect Dis 17: 366–371.
11. Mikota SK, Maslow JN (2011) Tuberculosis at the human-animal interface: an emerging disease of elephants. Tuberculosis (Edinb) 91: 208–211.
12. Keilty RA (1915) A Study of the Cultivation of the Tubercle Bacillus Directly from the Sputum by the Method of Petroff. J Exp Med 22: 612–614.
13. Chu LS (1955) Rapid method for cultivation of acid-fast bacilli. Science 122: 1189–1190.
14. Rachow A, Zumla A, Heinrich N, Rojas-Ponce G, Mtafya B, et al. (2011) Rapid and accurate detection of *Mycobacterium tuberculosis* in sputum samples by Cepheid Xpert MTB/RIF assay–a clinical validation study. PLoS One 6: e20458.
15. The National Tuberculosis Working Group for Zoo and Wildlife Species (2008) USDA. Guidelines for the control of tuberculosis in elephants, 2008. Available: http://www.aphis.usda.gov/animal_welfare/downloads/elephant/elephant_tb.pdf Accessed 2009 Jun 10
16. Moller T, Roken B, Petersson L, Vitaud C, Lyashchenko K (2005) Preliminary results of a new serological test for detection of TB-infection (*Mycobacterium tuberculosis*) in elephants (*Elephas maximus* and *Loxodonta africanum*) - Swedish Case studies. VerhberErkrgZootiere 42: 173–181.
17. Mikota SK, Peddie L, Peddie J, Isaza R, Dunker F, et al. (2001) Epidemiology and diagnosis of *Mycobacterium tuberculosis* in captive Asian elephants (*Elephas maximus*). J Zoo Wildl Med 32: 1–16.
18. Lewerin SS, Olsson SL, Eld K, Roken B, Ghebremichael S, et al. (2005) Outbreak of *Mycobacterium tuberculosis* infection among captive Asian elephants in a Swedish zoo. Vet Rec 156: 171–175.
19. Payeur JB, Jarnagin JL, Marquardt JG, Whipple DL (2002) Mycobacterial isolations in captive elephants in the United States. Ann N Y Acad Sci 969: 256–258.

20. Larsen RS, Salman MD, Mikota SK, Isaza R, Montali RJ, et al. (2000) Evaluation of a multiple-antigen enzyme-linked immunosorbent assay for detection of *Mycobacterium tuberculosis* infection in captive elephants. J Zoo Wildl Med 31: 291–302.
21. Lyashchenko KP, Greenwald R, Esfandiari J, Olsen JH, Ball R, et al. (2006) Tuberculosis in elephants: antibody responses to defined antigens of *Mycobacterium tuberculosis*, potential for early diagnosis, and monitoring of treatment. Clin Vaccine Immunol 13: 722–732.
22. Greenwald R, Lyashchenko O, Esfandiari J, Miller M, Mikota S, et al. (2009) Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants. Clin Vaccine Immunol 16: 605–612.
23. Lyashchenko KP, Greenwald R, Esfandiari J, Mikota S, Miller M, et al. (2012) Field Application of Serodiagnostics to Identify Elephants with Tuberculosis prior to Case Confirmation by Culture. Clin Vaccine Immunol.
24. Harboe M, Oettinger T, Wiker HG, Rosenkrands I, Andersen P (1996) Evidence for occurrence of the ESAT-6 protein in *Mycobacterium tuberculosis* and virulent *Mycobacterium bovis* and for its absence in *Mycobacterium bovis* BCG. Infect Immun 64: 16–22.
25. Skjot RL, Oettinger T, Rosenkrands I, Ravn P, Brock I, et al. (2000) Comparative evaluation of low-molecular-mass proteins from *Mycobacterium tuberculosis* identifies members of the ESAT-6 family as immunodominant T-cell antigens. Infect Immun 68: 214–220.
26. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, et al. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393: 537–544.
27. Chaturvedi R, Bansal K, Narayana Y, Kapoor N, Sukumar N, et al. (2010) The multifunctional PE_PGRS11 protein from *Mycobacterium tuberculosis* plays a role in regulating resistance to oxidative stress. J Biol Chem 285: 30389–30403.
28. Bansal K, Elluru SR, Narayana Y, Chaturvedi R, Patil SA, et al. (2010) PE_PGRS antigens of *Mycobacterium tuberculosis* induce maturation and activation of human dendritic cells. J Immunol 184: 3495–3504.
29. Alonzo TA, Pepe MS (1999) Using a combination of reference tests to assess the accuracy of a new diagnostic test. Stat Med 18: 2987–3003.
30. World Organization for Animal Health (OIE) (2010) Principles of validation of diagnostic assays for infectious diseases. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals OIE, Paris, France: 1–18.
31. TDR Diagnostics Evaluation Expert Panel, Banoo S, Bell D, Bossuyt P, Herring A, et al. (2010) Evaluation of diagnostic tests for infectious diseases: general principles. Nat Rev Microbiol 8: S17–29.
32. Rindskopf D, Rindskopf W (1986) The value of latent class analysis in medical diagnosis. Stat Med 5: 21–27.
33. Qu Y, Tan M, Kutner MH (1996) Random effects models in latent class analysis for evaluating accuracy of diagnostic tests. Biometrics 52: 797–810.
34. Pepe MS, Janes H (2007) Insights into latent class analysis of diagnostic test performance. Biostatistics 8: 474–484.
35. Boelaert M, Aoun K, Liinev J, Goetghebeur E, Van der Stuyft P (1999) The potential of latent class analysis in diagnostic test validation for canine *Leishmania infantum* infection. Epidemiol Infect 123: 499–506.
36. Frossling J, Bonnett B, Lindberg A, Bjorkman C (2003) Validation of a Neospora caninum iscom ELISA without a gold standard. Prev Vet Med 57: 141–153.
37. Rose N, Boutrouille A, Fablet C, Madec F, Eloit M, et al. (2010) The use of Bayesian methods for evaluating the performance of a virus-like particles-based ELISA for serology of hepatitis E virus infection in swine. J Virol Methods 163: 329–335.
38. Clegg TA, Duignan A, Whelan C, Gormley E, Good M, et al. (2011) Using latent class analysis to estimate the test characteristics of the gamma-interferon test, the single intradermal comparative tuberculin test and a multiplex immunoassay under Irish conditions. Vet Microbiol 151: 68–76.
39. Morton JM, McCoy RJ, Kann RK, Gardner IA, Meers J (2012) Validation of real-time polymerase chain reaction tests for diagnosing feline immunodeficiency virus infection in domestic cats using Bayesian latent class models. Prev Vet Med 104: 136–148.
40. Greiner M, Gardner IA (2000) Epidemiologic issues in the validation of veterinary diagnostic tests. Prev Vet Med 45: 3–22.
41. More SJ, Cameron AR, Greiner M, Clifton-Hadley RS, Rodeia SC, et al. (2009) Defining output-based standards to achieve and maintain tuberculosis freedom in farmed deer, with reference to member states of the European Union. Prev Vet Med 90: 254–267.
42. Pai M, Dendukuri N, Wang L, Joshi R, Kalantri S, et al. (2008) Improving the estimation of tuberculosis infection prevalence using T-cell-based assay and mixture models. Int J Tuberc Lung Dis 12: 895–902.
43. Dendukuri N, Hadgu A, Wang L (2009) Modeling conditional dependence between diagnostic tests: a multiple latent variable model. Stat Med 28: 441–461.
44. Kunnath-Velayudhan S, Salamon H, Wang HY, Davidow AL, Molina DM, et al. (2010) Dynamic antibody responses to the *Mycobacterium tuberculosis* proteome. Proc Natl Acad Sci U S A 107: 14703–14708.
45. Davidow A, Kanaujia GV, Shi L, Kaviar J, Guo X, et al. (2005) Antibody profiles characteristic of *Mycobacterium tuberculosis* infection state. Infect Immun 73: 6846–6851.
46. Kunnath-Velayudhan S, Gennaro ML (2011) Immunodiagnosis of tuberculosis: a dynamic view of biomarker discovery. Clin Microbiol Rev 24: 792–805.
47. Steingart KR, Dendukuri N, Henry M, Schiller I, Nahid P, et al. (2009) Performance of purified antigens for serodiagnosis of pulmonary tuberculosis: a meta-analysis. Clin Vaccine Immunol 16: 260–276.
48. Diel R, Loddenkemper R, Nienhaus A (2010) Evidence-based comparison of commercial interferon-gamma release assays for detecting active TB: a metaanalysis. Chest 137: 952–968.
49. Sester M, Sotgiu G, Lange C, Giehl C, Girardi E, et al. (2011) Interferon-gamma release assays for the diagnosis of active tuberculosis: a systematic review and meta-analysis. Eur Respir J 37: 100–111.
50. Steingart KR, Flores LL, Dendukuri N, Schiller I, Laal S, et al. (2011) Commercial serological tests for the diagnosis of active pulmonary and extrapulmonary tuberculosis: an updated systematic review and meta-analysis. PLoS Med 8: e1001062.
51. Arloing S (1898) Agglutination de bacille de la tuberculose vraie. Les Comptes Rendus de l'Academie des Sciences 126: 1398–1400.
52. Narayana Y, Joshi B, Katoch VM, Mishra KC, Balaji KN (2007) Differential B-cell responses are induced by *Mycobacterium tuberculosis* PE antigens Rv1169c, Rv0978c, and Rv1818c. Clin Vaccine Immunol 14: 1334–1341.
53. Espitia C, Laclette JP, Mondragon-Palomino M, Amador A, Campuzano J, et al. (1999) The PE-PGRS glycine-rich proteins of *Mycobacterium tuberculosis*: a new family of fibronectin-binding proteins? Microbiology 145 (Pt 12): 3487–3495.
54. Singh KK, Dong Y, Patibandla SA, McMurray DN, Arora VK, et al. (2005) Immunogenicity of the *Mycobacterium tuberculosis* PPE55 (Rv3347c) protein during incipient and clinical tuberculosis. Infect Immun 73: 5004–5014.
55. Aagaard C, Govaerts M, Meikle V, Vallecillo AJ, Gutierrez-Pabello JA, et al. (2006) Optimizing antigen cocktails for detection of *Mycobacterium bovis* in herds with different prevalences of bovine tuberculosis: ESAT6-CFP10 mixture shows optimal sensitivity and specificity. J Clin Microbiol 44: 4326–4335.
56. Ameni G, Aseffa A, Engers H, Young D, Hewinson G, et al. (2006) Cattle husbandry in Ethiopia is a predominant factor affecting the pathology of bovine tuberculosis and gamma interferon responses to mycobacterial antigens. Clin Vaccine Immunol 13: 1030–1036.
57. Lafferty K (2002) Good medicine for conservation biology: The intersection of epidemiology and conservation theory. Conservation Biology 16: 593–604.
58. Michel AL, Venter L, Espie IW, Coetzee ML (2003) *Mycobacterium tuberculosis* infections in eight species at the National Zoological Gardens of South Africa, 1991–2001. J Zoo Wildl Med 34: 364–370.
59. Kania SA, Richman LK, Kennedy MA, Montali RJ, Potgieter LND (1997) The isolation, Detection, and Cross-Reactivity of Asian Elephant IgG for the Development of Serological Diagnostic Tests. Veterinary Allergy Clinical Immunology 5: 125–128.
60. Kelly PJ, Carter SD, Azwai SM, Cadman HF (1998) Isolation and characterisation of immunoglobulin g and IgG subclasses of the African elephant (*Loxodonta africana*). Comp Immunol Microbiol Infect Dis 21: 65–73.
61. Guo Y, Bao Y, Wang H, Hu X, Zhao Z, et al. (2011) A preliminary analysis of the immunoglobulin genes in the African elephant (*Loxodonta africana*). PLoS One 6: e16889.
62. Archie E, Henry T, Maldonado J, Moss C, Poole J, et al. (2010) Major histocompatibility complex variation and evolution at a single, expressed DQA locus in two genera of elephants. Immunogenetics 62: 85–100.
63. Mushi EZ, Hill FWG, Dawe P, Riess R (1990) Antibodies to bluetongue and African horse sickness viruses in the sera of elephants in Zimbabwe. Bulletin of Animal Health and Production in Africa 38: 475.
64. Davies F, Otieno S (1977) Elephants and zebras as possible reservoir hosts for African horse sickness virus. Veterinary Record 100: 291–292.
65. Clark HW, Laughlin DC, Bailey JS, Brown TM (1980) Mycoplasma Species and Arthritis in Captive Elephants. The Journal of Zoo Animal Medicine 11: 3–15.
66. Rajan A, Vikram-Reddy M, Sulochana S, Valsala K (1980) Demonstration of T lymphocyte distribution in the peripheral blood of Indian elephant (*Elephas maximus*) using acid Alpha Napthyl Acetate Esterase activity as a T cell marker.. African Journal of Clinical Experimental Immunology 2: 357–362
67. Looringh van Beeck F, Reinink P, Hermsen R, Zajonc D, Laven M, et al. (2009) Functional CD1d and/or NKT cell invariant chain transcript in horse, pig, African elephant and guinea pig, but not in ruminants. Molecular Immunology 46: 1424–1431.
68. Khan IH, Ravindran R, Yee J, Ziman M, Lewinsohn DM, et al. (2008) Profiling antibodies to *Mycobacterium tuberculosis* by multiplex microbead suspension arrays for serodiagnosis of tuberculosis. Clin Vaccine Immunol 15: 433–438.
69. Abraham D, Davis J (2008) Revised Trunk was collection procedure for captive elephants in a range country setting. Gajah 28: 46–47.
70. A SK, Bansal K, Holla S, Verma-Kumar S, Sharma P, et al. (2012) ESAT-6 induced COX-2 expression involves coordinated interplay between PI3K and MAPK signaling. Mol Immunol 49: 655–663.
71. Manual AAL (1988) Protocols for Immunaffinity purification. In: Harlow E, Lane D, editors. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York. 519–554.
72. Macdonald PDM, Du J (2010) Mixdist: Finite Mixture Distribution Models. R package version 0.5–3. 2010.Available: http://cran.r-project.org/web/packages/mixdist/index.html. Accessed 2011 Sep 21

October 2013, Vol 144, No. 4_MeetingAbstracts
Chest Infections | October 2013

Cross-Species Tuberculosis Transmission: Two Probable Cases in Mahouts and Captive Elephants

Venugopal Kummannoor Parameswaran Pillai, MD; David Abraham, MPH

Government Medical College Hospital, Kottayam, India

Chest. 2013;144(4_MeetingAbstracts):215A. doi:10.1378/chest.1704012

Abstract

SESSION TITLE: Infectious Disease Global Case Reports

SESSION TYPE: Global Case Report

PRESENTED ON: Tuesday, October 29, 2013 at 01:30 PM - 02:30 PM

INTRODUCTION: In elephants, tuberculosis is a spillover disease resulting mostly from human cohabitation and most cases are caused by the human strains of Mycobacterium tuberculosis. Captive elephants in southern India are kept in hands-on contact with mahouts (elephant keepers) as well as public. In the field settings in southern India where there is a significant prevalence of tuberculosis in humans, human-to-elephant and elephant-to-human transmission of tuberculosis presents disease risks to both the in-contact species. A collaborative research involving medical physicians, veterinarians and molecular biologists is studying the transmission dynamics of tuberculosis infection among captive elephants and mahouts. Systematic tuberculosis screening of the nearly 1000 captive elephants and 5000 mahouts in southern India is underway. Screening of mahouts is done by clinical examination and tuberculin skin testing, followed by chest X-ray and sputum collection as required. Screening of elephants is done with the USDA licensed rapid serum tests Elephant TB STAT-PAK® and DPP Vet Assay® (Chembio Diagnostics Inc. Medford NY), followed by trunk wash culture for isolation and identification of mycobacteria.

CASE PRESENTATION: We identified two probable cases of cross-species transmission of M. tuberculosis between mahouts and captive elephants. First is case of human-to-elephant and second is a case of elephant-to-human transmission of M. tuberculosis. Case#1: A female elephant tested positive on Elephant TB STAT-PAK® and showed clinical symptoms of tuberculosis. There has been hand-on interaction between this elephant and its mahout for the past 25 years. The mahout was found to be suffering from active tuberculosis and tested positive on tuberculin skin test and showed shadows in his chest X-ray. M. tuberculosis culture isolate

was obtained from one of the three sputum samples collected from the mahout and was sensitive to first line anti-tuberculous drugs. Case#2: Another male elephant tested positive on Elephant TB STAT-PAK® and also showed symptoms of tuberculosis. The mahout associated with this elephant had a cutaneous nodule on the palm of right hand. This nodule, which was later operated and removed, was confirmed by histopathology as a case of tuberculous granuloma. Further inquiry revealed that this mahout previously suffered injuries to his hand while trying to control the elephant and the wound was contaminated with discharges from the elephant's trunk. The mahout tested positive on tuberculin skin test, but chest X-ray did not reveal any lesion suggestive of tuberculosis. Trunk wash samples from both elephants are collected for the isolation and identification of mycobacteria.

DISCUSSION: During the life span of nearly 50 years a captive elephant lives in close contact with humans. Also as a wild animal in captivity surviving in unnatural surroundings that add stress, the captive elephants present a unique animal model for an observational study to assess the disease risks of interspecies transmission of tuberculosis. This study intends to identify modifiable risk factors, if any, for effective policy intervention for prevention and control.

CONCLUSIONS: M. tuberculosis infection spillover and the risk of zoonosis from infected elephants is a well researched subject in the United States. In developing countries, mainly due to ineffective surveillance, such cases are rarely reported and documented. This poster presents a pictorial depiction of the different aspects of these two probable cases of cross-species transmission of M. tuberculosis.

Reference #1: Murphree, R., Warkentin, J. V., Dunn, J. R., Schaffner, W., & Jones, T. F. (2011). Elephant-to-human transmission of tuberculosis, 2009. Emerging Infectious Diseases, 17(3), 366-371.

Reference #2: Mikota, S. K., & Maslow, J. N. (2011). Tuberculosis at the human-animal interface: an emerging disease of elephants. Tuberculosis, 91(3), 208-211.

Reference #3: Greenwald, R., Lyashchenko, O., Esfandiari, J., Miller, M., Mikota, S., Olsen, J. H., . . . Moller, T. (2009). Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants. Clinical and Vaccine Immunology, 16(5), 605.

DISCLOSURE: The following authors have nothing to disclose: Venugopal Kummannoor Parameswaran Pillai, David Abraham

No Product/Research Disclosure Information

Type: Poster Presentation

Final Abstract Number: 43.244
Session: Poster Session III
Date: Saturday, March 5, 2016
Time: 12:45-14:15
Room: Hall 3 (Posters & Exhibition)

Viral burden in acute respiratory tract infections in hospitalized children in the wet and dry zones of Sri Lanka

J.A.A.S. Jayaweera[1], F. Noordeen[2,*], A. Morel[3], N. Pitchai[4], S. Kothalawala[5], A.M.S.B. Abeykoon[5], J.S.M. Peiris[6]

[1] *Faculty of Medicine and Allied Sciences Rajarata University Saliyapura Sri Lanka, Anuradhapura, Sri Lanka*
[2] *University of Peradeniya, Kandy, Sri Lanka*
[3] *Teaching Hospital Gampola Sri Lanka, Kandy, Sri Lanka*
[4] *Faculty of Medicine University of Peradeniya, Kandy, Sri Lanka*
[5] *Faculty of Medicine University of Peradeniya, Peradeniya, Sri Lanka*
[6] *The University Of Hong Kong, Hong Kong, Sri Lanka*

Background: Acute respiratory tract infection (ARTI) is one of the most common acute illnesses of childhood. Mostly encountered viral etiology of ARTI in children under 5 years are respiratory syncytial virus (RSV), parainfluenza types 1, 2 and 3 (PIV), adenovirus (AV), influenza virus types A and B, coronavirus (CoV), human Boca virus (hBoV) and human metapeumo virus. (hMPV) This study was conducted to identify the viral burden in hospitalized children with ARTIs to map the occurrence of these viruses with local seasonality.

Methods & Materials: Nasopharyngeal aspirates (NPA) of inward patients (1 month - 5 years) with ARTI were collected in Teaching Hospital, Gampola (THG) and Teaching Hospital, Anuradhapura (THA) from March 2013 - August 2014. Following screening of NPA with indirect immunofluorescence assay (IFA) specific viral aetiology was detected by a direct immunofluorescence assay (DFA). IFA negative hundred NPA were tested for hMPV, hBoV and CoV. Viral seasonality and the overall viral burden were evaluated and the descriptive statistics was expressed using measures of central tendency.

Results: Out of 443 and 418 NPAs tested, RSV was detected 94 children (59.96%) in THG and 85 children (51.51%) in THA. In both cohorts RSV was detected throughout the year. In the dry zone, the peak viral incidence was noted from May-July in 2013 and 2014. In the wet zone two peaks were observed: December-January in 2013 (major peak) and in April in 2013 and 2014 (minor peak). Period prevalence of RSV ARTI in THG was 4.7% and in THA was 4.25%. The RSV incidence at THG and THA was 31.3 and 28 /100000 person years. The hMPV distribution was similar to that of RSV.

Conclusion: Knowledge of seasonality of the occurrence of viral aetiologies in children with ARTI is important to implement early preventive measures, such as vaccination for influenza A, use of respiratory precautions and health education. Identifying the viral aetiology by proper virological diagnosis will reduce the empirical use of antibiotics and thus will contribute to reduce the cost and to prevent the emergence of anti-microbial resistance.

http://dx.doi.org/10.1016/j.ijid.2016.02.980

Type: Poster Presentation

Final Abstract Number: 43.245
Session: Poster Session III
Date: Saturday, March 5, 2016
Time: 12:45-14:15
Room: Hall 3 (Posters & Exhibition)

Cross-species transmission of mycobacterium tuberculosis in mahouts and captive elephants: Implications to health policy

D. Abraham[1,*], V. Kummannoor Parameswaran Pillai[2]

[1] *Theekuni Veterinary Dispensary, Thrissur, Kerala, India*
[2] *Government Medical College Hospital, Kottayam, Kerala, India, Kottayam, India*

Background: There are nearly a thousand captive Asian elephants and not less than 3,000 mahouts in southern India. In the hands-on and open systems of captive elephant management, diseased mahouts and captive elephants could present the risk of cross-species tuberculosis transmission. With the help of evidence based results, we intend to formulate specific policy guidelines, which can suggest locally relevant preventive and control measures to help mitigate the risk of cross-species infection.

Methods & Materials: Over a period of three years, one time screening of nearly 800 elephants and their mahouts was achieved. Tuberculosis screening of mahouts was done by clinical examination, chest X-ray evaluation, sputum culture and tuberculin skin testing, as required. Screening of elephants was done using the USDA licensed serological test, DPP Vet Assay® (Chembio Diagnostics Inc., Medford, New York) and trunk wash culture, as required. Detailed contact investigation of traceable human and animal contacts of the identified diseased mahouts and elephants were done. We examined three different contexts of tuberculosis transmission among captive elephants and mahouts. First scenario is the risk of infection from an infected mahout to an elephant. Second is the risk of infection from an infected elephant to a mahout and third is the risk of infection from an infected elephant to another elephant.

Results: There is evidence to suggest cross-species tuberculosis transmission. However, under the tropical climatic conditions in southern India, the risk of infection to a captive elephant from a diseased mahout seems to far outweigh the risks of infection to a mahout or another elephant, from a diseased elephant. There are political as well as ethical consequences to the outcomes in each of the three scenarios and they are both varied and complex.

Conclusion: Mahouts and captive elephants in southern India are highly migrant and locating the subjects for contact tracing and follow-up testing is difficult. Hence, systematic and regular tuberculosis screening of mahouts and captive elephants is a challenge. Formulating as well as implementing policy guidelines for prevention and control of cross-species tuberculosis transmission, in the existing cultural and religious contexts of captive elephant managements in southern India, appears to be an even bigger challenge.

http://dx.doi.org/10.1016/j.ijid.2016.02.981

ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/313366428

Mycobacterium tuberculosis in Wild Asian Elephants, Southern India

Article *in* Emerging Infectious Diseases · March 2017

DOI: 10.3201/eid2303.161741

CITATIONS
13

READS
289

8 authors, including:

Arun Zachariah
Kerala Veterinary and Animal Sciences University
46 PUBLICATIONS **145** CITATIONS
SEE PROFILE

Gk Madhavilatha
Rajiv Gandhi Centre for Biotechnology
82 PUBLICATIONS **69** CITATIONS
SEE PROFILE

Chandramohan Bathrachalam
SciGenom Research Foundation
49 PUBLICATIONS **79** CITATIONS
SEE PROFILE

Some of the authors of this publication are also working on these related projects:

Indian cattle WGS View project

Disease Ecology in Free Ranging Asian Elephants View project

All content following this page was uploaded by Chandramohan Bathrachalam on 21 February 2017.

The user has requested enhancement of the downloaded file.

Mycobacterium tuberculosis in Wild Asian Elephants, Southern India

Arun Zachariah, Jeganathan Pandiyan, G.K. Madhavilatha, Sathish Mundayoor, Bathrachalam Chandramohan, P.K. Sajesh, Sam Santhosh, Susan K. Mikota

We tested wild Asian elephants (*Elephas maximus*) in southern India and confirmed infection in 3 animals with *Mycobacterium tuberculosis,* an obligate human pathogen, by PCR and genetic sequencing. Our results indicate that tuberculosis may be spilling over from humans (reverse zoonosis) and emerging in wild elephants.

Infection with *Mycobacterium tuberculosis* in domestic and wild animals of various species living in close contact with humans has been reported (*1*). Elephants in captivity are known to be susceptible to infection with *M. tuberculosis*, and there is a potential for transmission of *M. tuberculosis* between humans and elephants (*2–4*). In 2013, a case of tuberculosis (TB) in a wild elephant in Africa, which had been under human care, was reported (*5*), after which another case in a wild Asian elephant in Sri Lanka was reported (*6*). Habitat encroachment and competition for resources brings wild elephants into closer contact with humans, providing opportunities for zoonoses and reverse zoonoses to occur and for a previously unknown pathogen to emerge in captive free-ranging and wild elephant populations.

The Study

In March 2007, an emaciated wild bull elephant, estimated to be 20 years of age, died shortly after it was found recumbent in the Muthanga range of the Wayanad Wildlife Sanctuary in southern India (case 1). Postmortem examination revealed purulent exudates throughout the lungs, an enlarged liver, enlarged mesenteric lymph nodes, and surface nodules containing caseated yellowish-white material (Figure 1). We found serosanguinous fluid in the pericardial sac and slightly hypertrophied heart ventricles. We saw focal areas of necrosis in the renal cortices but noted no other gross lesions. Ziehl-Neelsen staining of lung, liver, kidney, and mesenteric lymph node impression smears revealed numerous acid-fast bacilli. We confirmed the presence of *M. tuberculosis* by using PCR amplification of the targeted bacterial genome, gel documentation of the amplified products, and sequencing.

Subsequently, a surveillance program was initiated (until March 2014), and all fresh elephant carcasses in the study area were examined for evidence of TB (n = 88). In May 2010, a bull elephant, ≈30 years of age, was found dead in the Kurichiyat range (case 2). Postmortem examination revealed extensive caseated lesions in the lungs (Figure 2) and mild mesenteric lymph node hypertrophy. In May 2013, TB infection was diagnosed in a bull ≈40 years of age that was found in the same forest range and had extensive caseated lung lesions (case 3). Both bulls were emaciated.

We fixed samples for histopathological studies in 10% formol saline and embedded them in paraffin. We found numerous acid-fast organisms in lung impression smears and tissue sections. Granulomatous lesions encapsulated by connective tissue with aggregated macrophages and central areas of necrosis were seen during histopathologic examination of the lungs for all 3 cases and of the kidney and liver in case 1. Langerhans-type giant cells were observed in cases 2 and 3 but not in case 1.

Tissues for molecular studies were collected in absolute alcohol. We extracted total DNA from tissues by using DNeasy Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. DNA was subjected to a tetraplex PCR to differentiate between *M. tuberculosis* complex and nontuberculous mycobacteria. DNA was subjected to amplification and sequencing of the 3 target regions separately, 16S–23S internal transcribed spacer region, hsp65, and rpoB separately (*7*). *M. tuberculosis* H37Rv and *M. bovis* bacilli Calmette-Guérin genomic DNA was used as control DNA for the PCR studies.

We observed the expected 4-band pattern after tetraplex PCR. As the MTP40 fragment was amplified, *M. bovis* was ruled out because the *plcA* gene (mtp40), one of the members of the *plc* family of genes that code for the phospholipase C enzyme, is deleted in the *M. bovis* and *M. bovis* bacilli Calmette-Guérin RD5 region (*8*). Sequences that were generated were assembled and

Author affiliations: Kerala Veterinary and Animal Sciences University, Kerala, India (A. Zachariah); AVC College, Tamil Nadu, India (J. Pandiyan); Rajiv Gandhi Centre for Biotechnology, Kerala (G.K.Madhavilatha, S. Mundayoor); Scigenom Research Foundation, Kerala (B. Chandramohan, P.K. Sajesh, S. Santhosh); Elephant Care International, Hohenwald, Tennessee, USA (S.K. Mikota)

DOI: http://dx.doi.org/10.3201/eid2303.161741

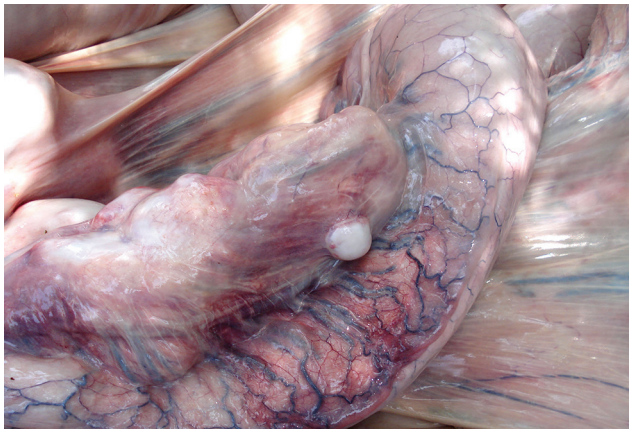


Figure 1. Intestine from a wild bull elephant, estimated at 20 years of age, Wayanad Wildlife Sanctuary, India, 2007. Multiple white-to-tan discrete nodules (granulomas) are protruding from the serosal surface, and less well-defined areas of pale discoloration are visible within the intestinal wall. Serosal blood vessels are markedly dilated, tortuous, and congested

edited by using the alignment software Seqscape (http://www.seqscape.software.informer.com). BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) analysis of the edited sequences revealed that the elephant sequences showed 100% similarity with the *M. tuberculosis* genome fragment. We also used DNA for large sequence polymorphism analysis to determine the lineage of *M. tuberculosis* using RD239 and RD750 primers (*9,10*). The genomic deletion analysis revealed a deletion in RD239, which is characteristic of the Indo-Oceanic lineage (*10*), also referred to as the East African–Indian lineage (*11*).

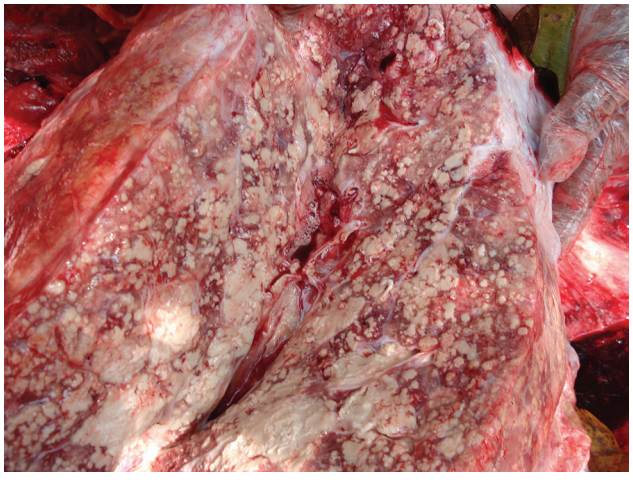


Figure 2. Lung from a bull elephant, estimated at 30 years of age, Kurichiyat Range, India, 2010. Note the multifocal to coalescing pale tan-to-white firm nodules (granulomas) effacing much of the lung parenchyma. Some areas of white chalky mineralization are also present.

Conclusions

There are reports of mycobacterial infections in captive elephants in India from as early as 1925 (*12*). We report *M. tuberculosis* infection in wild elephants in India. In this study, 3 (3.4%) of 88 elephants undergoing postmortem examination were confirmed to be infected with *M. tuberculosis*. All 3 animals were emaciated, and we considered TB to be the cause of death.

The close interaction between humans and captive elephants is presumed to be a key risk factor for the interspecies transmission of TB. The epidemiology of TB among wild elephants, now documented in 3 countries, has yet to be elucidated. In our study, there were no known captive elephant releases or reintroductions into the study area, and the interaction between captive and wild elephants is considered negligible. However, native tribes do live within the park; many tribal members are employed by the forest department for protection and ecotourism activities. Tourists may visit specified areas only under supervision; there are no overnight facilities. Human–elephant conflict is a problem; most conflicts are caused by resident bulls. All 3 TB cases reported here were in bulls. Exposure of bulls to humans infected with TB during conflict activities is a possible explanation.

More than 3,000 native cattle reside within the sanctuary, cared for by the Animal Husbandry Department, Kerala State. No cases of TB among cattle have been reported. Cattle would be more likely to be infected with *M. bovis* than with *M. tuberculosis*, but comprehensive testing would be informative. Cattle living in close proximity to TB-infected humans can become infected with *M. tuberculosis* (*13*). Whether such infected cattle could then transmit *M. tuberculosis* to elephants through contamination of shared grazing lands is yet another research question.

The *M. tuberculosis* complex is thought to have emerged as a human pathogen in Africa rather than arising from an animal source (*14*). Although the epidemiology has not been defined, our study and previous reports indicate that *M. tuberculosis* appears to be spilling over into elephants (reverse zoonosis) and emerging among wild elephant populations. Although these cases may have resulted from individual introductions, if *M. tuberculosis* becomes established, wild elephants and other susceptible species will be at risk.

Ecologic, environmental, or demographic factors that place animals or humans at increased contact can contribute to disease emergence. Certainly, the increased human–elephant conflict in India and other Asian elephant range countries attests to the narrowing interface between humans and elephants. This study suggests that *M. tuberculosis* is emerging in the largest single population of Asian elephants in India. Continued surveillance in India and other Asian elephant range countries is warranted.

Acknowledgments

We acknowledge permission from Kerala State Forest Department for conducting postmortem examinations in elephants in the Wayanad Wildlife Sanctuary.

This work was funded through a grant from US Fish and Wildlife Services, Asian Elephant Conservation Fund grant no. ASE 0491, and partial funding from the Scigenom Research Foundation.

The work was done at the Centre for Wildlife Studies, Kerala Veterinary and Animal Sciences University.

Dr. Zachariah is the assistant forest veterinary officer in the Department of Forests and Wildlife, Government of Kerala, India. His research interests are wildlife disease ecology, host–pathogen coevolution, and disease spillover among wildlife, humans, and domestic livestock.

References

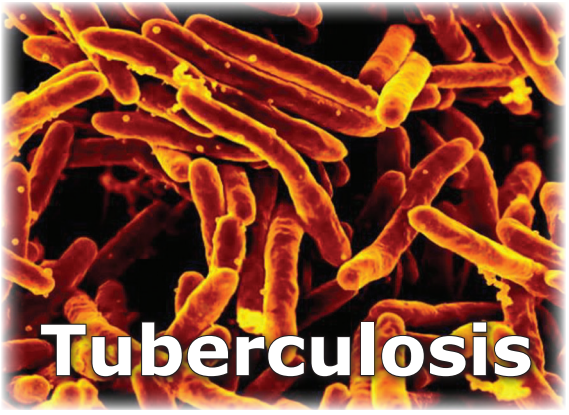
1. Montali RJ, Mikota SK, Cheng LI. *Mycobacterium tuberculosis* in zoo and wildlife species. Rev Sci Tech. 2001;20:291–303. http://dx.doi.org/10.20506/rst.20.1.1268
2. Michalak K, Austin C, Diesel S, Bacon MJ, Zimmerman P, Maslow JN. *Mycobacterium tuberculosis* infection as a zoonotic disease: transmission between humans and elephants. Emerg Infect Dis. 1998;4:283–7. http://dx.doi.org/10.3201/eid0402.980217
3. Mikota SK, Maslow JN. Tuberculosis at the human-animal interface: an emerging disease of elephants. Tuberculosis (Edinb). 2011;91:208–11. http://dx.doi.org/10.1016/j.tube.2011.02.007
4. Murphree R, Warkentin JV, Dunn JR, Schaffner W, Jones TF. Elephant-to-human transmission of tuberculosis, 2009. Emerg Infect Dis. 2011;17:366–71. http://dx.doi.org/10.3201/eid1703.101668
5. Obanda V, Poghon J, Yongo M, Mulei I, Ngotho M, Waititu K, et al. First reported case of fatal tuberculosis in a wild African elephant with past human-wildlife contact. Epidemiol Infect. 2013;141:1476–80. http://dx.doi.org/10.1017/S0950268813000022
6. Perera BVP, Salgadu MA, Gunawardena GSPdeS, Smith NH, Jinadasa HRN. First confirmed case of fatal tuberculosis in a wild Sri Lankan elephant. Gajah. 2013;41:28–31.
7. Anilkumar AK, Madhavilatha GK, Paul LK, Radhakrishnan I, Kumar RA, Mundayoor S. Standardization and evaluation of a tetraplex polymerase chain reaction to detect and differentiate *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria—a retrospective study on pulmonary TB patients. Diagn Microbiol Infect Dis. 2012;72:239–47. http://dx.doi.org/10.1016/j.diagmicrobio.2011.11.006
8. Gordon SV, Brosch R, Billault A, Garnier T, Eiglmeier K, Cole ST. Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome arrays. Mol Microbiol. 1999;32:643–55. http://dx.doi.org/10.1046/j.1365-2958.1999.01383.x
9. Tsolaki AG, Hirsh AE, DeRiemer K, Enciso JA, Wong MZ, Hannan M, et al. Functional and evolutionary genomics of *Mycobacterium tuberculosis:* insights from genomic deletions in 100 strains. Proc Natl Acad Sci U S A. 2004;101:4865–70. http://dx.doi.org/10.1073/pnas.0305634101
10. Gagneux S, DeRiemer K, Van T, Kato-Maeda M, de Jong BC, Narayanan S, et al. Variable host-pathogen compatibility in *Mycobacterium tuberculosis.* Proc Natl Acad Sci U S A. 2006;103:2869–73. http://dx.doi.org/10.1073/pnas.0511240103
11. Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, et al. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol. 2006;6:23. http://dx.doi.org/10.1186/1471-2180-6-23
12. Narayanan RS. A case of tuberculosis in an elephant. J Comp Pathol. 1925;38:96–7. http://dx.doi.org/10.1016/S0368-1742(25)80016-X
13. Ocepek M, Pate M, Zolnir-Dovc M, Poljak M. Transmission of *Mycobacterium tuberculosis* from human to cattle. J Clin Microbiol. 2005;43:3555–7. http://dx.doi.org/10.1128/JCM.43.7.3555-3557.2005
14. Comas I, Coscolla M, Luo T, Borrell S, Holt KE, Kato-Maeda M, et al. Out-of-Africa migration and Neolithic coexpansion of *Mycobacterium tuberculosis* with modern humans. Nat Genet. 2013;45:1176–82. http://dx.doi.org/10.1038/ng.2744

Address for correspondence: Susan K. Mikota, Elephant Care International, 166 Limo View Ln, Hohenwald, TN 38462, USA; email: smikota@elephantcare.org

EID SPOTLIGHT TOPIC

World TB Day, falling on March 24th each year, is designed to build public awareness that tuberculosis today remains an epidemic in much of the world, causing the deaths of nearly one-and-a-half million people each year, mostly in developing countries. It commemorates the day in 1882 when Dr Robert Koch astounded the scientific community by announcing that he had discovered the cause of tuberculosis, the TB bacillus. At the time of Koch's announcement in Berlin, TB was raging through Europe and the Americas, causing the death of one out of every seven people. Koch's discovery opened the way towards diagnosing and curing TB.

Click on the link below to access *Emerging Infectious Diseases* articles and podcasts, and to learn more about the latest information and emerging trends in TB.



EMERGING INFECTIOUS DISEASES® **http://wwwnc.cdc.gov/eid/page/world-tb-day**

View publication stats