Implementation of minimally invasive and objective humane endpoints in the study of murine *Plasmodium* infections

B. DELLAVALLE[1,2]*, J. KIRCHHOFF[1,2], L. MARETTY[1,2], F. C. CASTBERG[1,2] *and* J. A. L. KURTZHALS[1,2]

[1] *Centre for Medical Parasitology, Department of Clinical Microbiology, Copenhagen University Hospital, Copenhagen, Denmark*
[2] *Department of International Health, Immunology, and Microbiology, University of Copenhagen, Copenhagen, Denmark*

(*Received 25 February 2014; revised 14 April 2014; accepted 19 April 2014; first published online 4 July 2014*)

SUMMARY

Defining appropriate and objective endpoints for animal research can be difficult. Previously we evaluated and implemented a body temperature (BT) of <32 °C as an endpoint for experimental cerebral malaria (ECM) and were interested in a similar endpoint for a model of severe malarial anaemia (SMA). Furthermore, we investigate the potential of a minimally invasive, non-contact infrared thermometer for repeated BT measurement. ECM was induced with *Plasmodium berghei* ANKA infection in C57Bl/6 mice. SMA was induced with *Plasmodium chabaudi* AS infection in A/J mice. Our previous published endpoint was applied in ECM and 30 °C was pre-determined as the lowest permitted limit for termination in SMA according to consultation with the Danish Animal Inspectorate. Infrared thermometer was compared with the rectal probe after cervical dislocation, ECM and SMA. Linear regression analysis of rectal versus infrared thermometry: cervical dislocation: Pearson $R = 0{\cdot}99$, $R^2 = 0{\cdot}98$, slope = 1·01, y-intercept = 0·55; ECM: 0·99, 0·98, 1·06, −2·4; and SMA: 0·98, 0·97, 1·14, −5·6. Implementation of the 30 °C endpoint captured all lethal infections. However, some animals with BT below 30 °C were not deemed clinically moribund. This study supports repeated measurement infrared thermometry. A humane endpoint of 30 °C was sensitive in capturing terminal animals but might overestimate lethality in this SMA model.

Key words: *Plasmodium*, humane endpoints, infrared body temperature, cerebral malaria, malarial anaemia.

INTRODUCTION

Ethical standards generally require termination of animal experiments before obvious suffering of experimental animals. This precludes survival studies from using actual death as the readout. The definition of appropriate and objective humane endpoints has thus become a pervasive problem for improving the ethical standard in animal experimentation. In particular, defining objective and robust endpoints for infectious disease research has proven difficult. However, since infectious disease in humans can often be acute, aggressive and deadly, survival is a valuable readout for testing of e.g. potential drug candidates. Objectivity can be considered a golden standard in humane endpoint measurement; however, many animal models still lack unbiased and reliable objective endpoints. This complicates the use and comparison of survival studies. Additionally, with the progression of technology and 'out-of-animal' research techniques, animal inspectorates and the public at large are increasing the demand for objective and humane measures. In our research on experimental cerebral malaria (ECM), we proposed the use of a core body temperature (BT) below 32 °C as an objective predictor of morbidity and death in the classical *Plasmodium berghei* ANKA/C57Bl/6j ECM model (Wiese *et al*. 2008). Furthermore, BT has been proposed as an objective humane endpoint in models of sepsis, viral and fungal infections in the past (Soothill *et al*. 1992; Wong *et al*. 1997; Stiles *et al*. 1999; Warn *et al*. 2003; Nemzek *et al*. 2004). In our efforts to establish a lethal model of severe malarial anaemia (SMA), we opted to use the *Plasmodium chabaudi* AS-infected A/J mice model as described earlier by Yap and Stevenson (1992) in order to evaluate hypotheses concerning erythropoeitic suppression in future studies. In order to assess lethality we wished to implement BT as an objective humane endpoint for this model that has – to our knowledge – not been previously described.

Furthermore, repeated endpoint assessments are required to adhere to humane endpoints in a timely manner. We have recently been concerned with the invasive nature of repeated rectal probe measurement in our assessment of animal well-being, as it carries a risk of intestinal perforation. We therefore wished to assess the feasibility of minimally invasive BT measurements through use of non-contact infrared emission thermometers. This would allow us to take more frequent measurements of our endpoint without increasing distress and complications associated with acquiring this measurement. However, this

* Corresponding author: KMA, Copenhagen University Hospital 76·02, Tagensvej 20, Copenhagen N, 2200, Denmark. E-mail: briandellavalle@gmail.com

Parasitology (2014), **141**, 1621–1627.
doi:10.1017/S0031182014000821

method must be able to robustly reproduce core BT measurements from rectal thermometers, especially in compromised animals with vascular and neurological distress.

In this study we present analysis of the implementation of BT <30 °C as a potential objective endpoint for the study of SMA with a lethal *Plasmodium chaubaudi* AS infection. Additionally, we study if the minimally invasive infrared-emission measurement can reliably measure BT in mice in comparison with the rectal probe.

MATERIALS AND METHODS

Animals and ethics

All animals were kept under standard conditions in a closed, ventilated rack system with food/water access *ad libitum*. All studies were conducted to minimize suffering and in accordance with the Danish Animal Experiments Inspectorate according to licence 2012-15-2934-00449.

Infrared-emission measurement

In the initial testing, an infrared thermometer (845, Testo, DE) was tested against rectal probe (DM852, Ellab, DK) measurement to determine, calibrate and standardize the emission index and the method of handling. Animals were restrained by the neck skin and tail. Measurements were obtained by tracing the epigastral region and the maximum registered temperature was reported. This region was chosen based on pilot experiments in other regions of the mouse body (head, back, abdominal) where temperatures did not report similar to core body temperature. An emission index of 0·75 was determined to be ideal and adopted throughout the study. Emissivity is a measure of efficiency of an object to emit thermal energy relative to a theoretical 'perfect' black-body emitter (emissivity = 1·0).

Cervical dislocation

Investigator #1 determined the BT of C57Bl/6 mice ($N = 5$) with infrared thermometer. Blinded to the infrared measurement, investigator #2 inserted the rectal probe to a pre-defined depth. Thereafter, anaesthesia was induced with i.p. hypnorm/dormicum and animals were killed by cervical dislocation. Infrared and rectal probe measurements were repeated every 5 min until BT dropped below 29 °C.

Plasmodium berghei *cerebral malaria*. Throughout a previously reported study (DellaValle *et al*. 2013) we tested the use of the infrared thermometer in comparison with the rectal probe. In brief, *P. berghei* ANKA parasites were thawed from a previously characterized batch of infected red blood cells and injected i.p. into a pilot C57Bl/6 mouse (Taconic, DK). After 3 days' incubation in the host, 10^4 parasites were transferred to female C57Bl/6 mice age 6–10 weeks ($N = 14$) that received 100 μl 0·9% saline (placebo) as part of the study. Animals were monitored for temperature first with infrared thermometer and subsequently with rectal probe to a pre-defined depth from day 4 of infection until they dropped below the predetermined humane endpoint of 32 °C. ECM was diagnosed based on increasing parasitaemia, neurological signs, reduced BT and post-mortem observation of cerebral haemorrhaging as described (DellaValle *et al*. 2013). Two animals did not develop ECM and were thus excluded.

Plasmodium chabaudi *malarial anaemia infection*. In order to establish a partially lethal model of *P. chabaudi* AS infection in A/J mice (Harlan, NL) discussions were held with the Danish Animal Inspectorate. Based on these discussions a pilot study was conducted comparing increasing parasite loads (10^4–10^6, $N = 5$ per inoculum). A composite humane endpoint would be applied with observation of clinical presentation (fur appearance, home cage locomotion, grooming behaviour, urine colouration), weight loss and rectal BT measurement with an agreed lower limit of 30 °C. Mice with a BT below 34 °C were monitored three times daily. Thus, animals with a sharp decline in clinical health and/or measured below 30 °C were considered terminal. SMA was verified with increasing parasitaemia and decreasing haemoglobin. Animals given 10^4 parasites had 100% survival, 10^5 parasites had a 40% survival whereas 10^6 had a 0% survival (Kirchhoff *et al*. unpublished data). Additionally, at 10^5 no spontaneous deaths occurred. Based on these observations, the parasite load of 10^5 was adopted as a partially lethal inoculum and the lower limit of 30 °C was implemented as a humane predictor of lethality.

Infrared thermometer testing

To test the applicability of the infrared thermometer in SMA of A/J mice, in an independent study, animals were monitored for BT first with the infrared thermometer and thereafter with the rectal probe inserted to a pre-defined depth at baseline before infection and again once the animals showed signs of distress and BT temperature reduction ($N = 96$ measurements). SMA was confirmed with increasing parasitaemia and decreasing haemoglobin (Kirchhoff *et al*. unpublished data).

Implementation of 30 °C humane endpoint

Forty A/J mice were infected i.p. with 10^5 *P. chabaudi* parasites after passage in a pilot mouse in an independent study. Animals were monitored with

https://doi.org/10.1017/S0031182014000821 Published online by Cambridge University Press

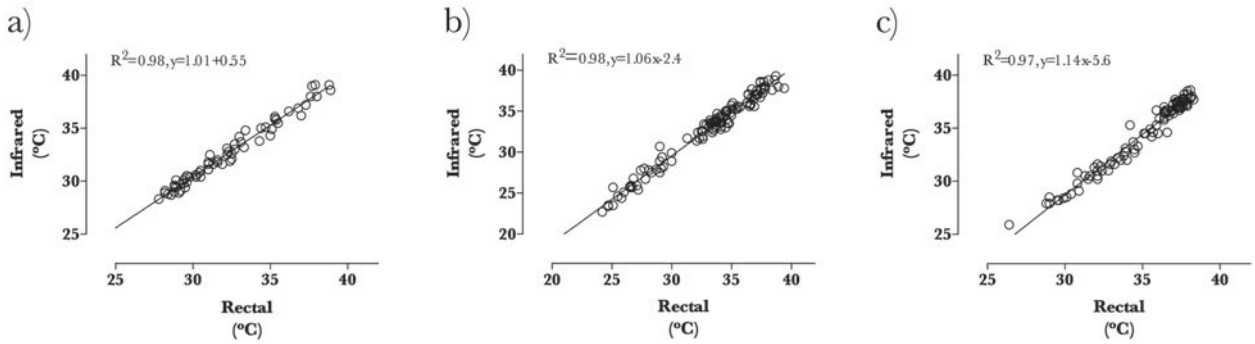

Fig. 1. Linear regression analysis comparing infrared and rectal thermometers in mice. A non-contact infrared thermometer recorded body temperature in the epigastral region of restrained mice. Thereafter a rectal probe measurement was taken at a pre-defined depth. Body temperature measurements for each instrument are presented in a scatter plot after (a) cervical dislocation, (b) infection with *Plasmodium berghei* ANKA and (c) infection with *P. chabaudi* AS. Pearson R^2 coefficient and slope are presented for each condition. (a) $N = 4$ animals, 69 measurements; (b) $N = 12$ animals, 114 measurements; (c) $N = 48$ animals, 96 measurements.

the infrared thermometer throughout the experiment and were observed for clinical symptoms and distress throughout the experiment (once daily until clinical presentation, three times daily thereafter). Animals with an infrared reading below 30 °C were only deemed terminal when BT was verified with the rectal thermometer. SMA was confirmed with increasing parasitaemia and decreasing haemoglobin. 'Moribund' was used to describe animals with ruffled fur, little to no motion, slow or no avoidance to touch and clear cold sensation to touch.

Determination of parasitaemia and haemoglobin in blood

Methods are described in detail in (Hein-Kristensen *et al*. 2009; Maretty *et al*. 2012; DellaValle *et al*. 2013). Briefly, for estimation of parasitaemia 2 μL of tail-vein blood was suspended in heparinized PBS, diluted further in PBS and 0·5 μg mL^{-1} acridine orange (Sigma-Aldrich, Germany), incubated in the dark for 30 min and counted on a BD FACSCanto (BD Biosciences, USA). Data were analysed with FlowJo (TreeStar INc., OR, USA) through gating of erythrocytes, and infected erythrocytes based on DNA/RNA content. Monomeric haemoglobin concentration was determined using AHD-575 Spectrophotometry on 2 μL of tail-vein blood mixed with 248 μL of 0·1 M NaOH with 2·5% Triton X-100 (Sigma-Aldrich, DE) in doublet. Absorbance measured at 595 nm on a Thermo Labsystems Multiscan EX photometer (ThermoScientific, DK) and haemoglobin was calculated using a standard curve.

Statistical analysis

Data were tested for normality (Shapiro–Wilk). Linear regression analysis was performed with rectal probe as independent and infrared as dependent variables, respectively. Pearson R (95% confidence interval) and R^2 values are reported for parametric data (BT) along with the slope and y-intercept. For non-parametric data (haemoglobin concentration and parasitaemia) Spearman's R is provided. Parametric data are presented as mean + S.E.M. and non-parametric as median + 75% percentile.

RESULTS

Performance of minimally invasive infrared thermometer

In our first set of experiments we compared the proposed infrared device in repeated measurements against rectal probe in animals as BT dropped after cervical dislocation. The performance of the infrared thermometer strongly correlated to the rectal readings (Pearson $R = 0{\cdot}99$ [0·98–0·99], $R^2 = 0{\cdot}98$, slope = 1·01, y-intercept = 0·55; $N = 67$ measurements; Fig. 1a).

We were thereafter interested in implementing the use of infrared measurement in our ECM experiments as a substitute for rectal probe measurement. Thus, the performance of the infrared thermometer was tested against rectal measurements in ECM study with a pre-defined humane endpoint of 32 °C. Similarly to the cervical dislocation experiment, the infrared performance corresponded strongly to the rectal measurements (Pearson $R = 0{\cdot}99$ [0·98–0·99], $R^2 = 0{\cdot}98$, slope = 1·06, y.intercept = −2·4, Fig. 1b; $N = 112$ measurements).

Similarly to previous experiments with cervical dislocation and ECM, the minimally invasive infrared thermometer performed strongly in SMA, correlating well with rectal probe measurements (Pearson $R = 0{\cdot}98$ [0·98–0·99], $R^2 = 0{\cdot}97$, slope = 1·14, y-intercept = −5·6; Fig. 1c; $N = 96$ measurements).

The differences between rectal and infrared BT measurements were −0·4 ± 0·06 °C; 0·2 ± 0·07 °C; 0·6 ± 0·07 °C for cervical dislocation, ECM and SMA animals, respectively (Appendix: Fig. A1).

https://doi.org/10.1017/S0031182014000821 Published online by Cambridge University Press

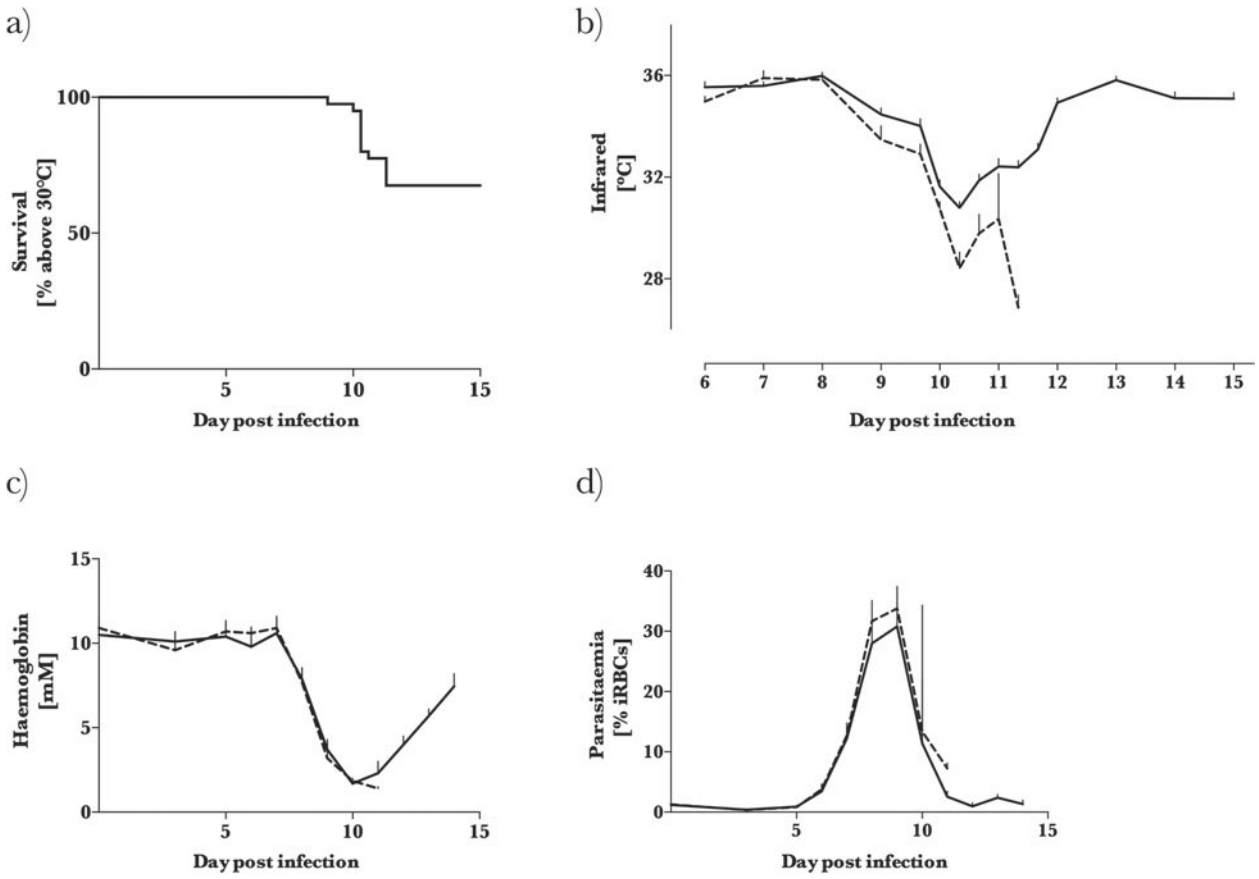

Fig. 2. Infrared body temperature and survival in *P. chabaudi* infection of A/J mice. A/J mice ($N = 40$) were injected i.p. with 10^5 *P. chabaudi* AS parasites in a murine model of malarial anaemia. Animals were measured for body temperature with a non-contact infrared thermometer in the epigastral region once daily until clinical symptoms were present. Thereafter, mice were recorded three times daily to monitor for reduced body temperature. Animals that dropped below the pre-defined humane endpoint of 30 °C were measured for rectal body temperature. Confirmed measurements below 30 °C were deemed terminal. Animals were measured for haemoglobin and parasitaemia throughout the experiment. (a) Survival curve is based on body temperature drops 30 °C presented as a Kaplan–Meier plot. No unexpected deaths occurred and all mice developed malarial anaemia. (b) Body temperature data were normal and thus reported as mean + S.E.M. for surviving mice (bold line) and terminal mice (dotted line) based on whether any given mouse dropped below 30 °C (c). Haemoglobin (c) and parasitaemia (d) data were not normally distributed and are presented as median + 75% percentile and are separated into surviving mice (bold line) and terminal mice (dotted line) based on whether any given mouse dropped below 30 °C. (N = surviving: 27, terminal: 13). (Total $N = 40$).

In all conditions BT measurements varied above and below the zero-difference value.

Applying an objective humane endpoint in P. chabaudi *infection.* A suitable endpoint applicable for acute disease models is, ideally, directly available to the researcher in the animal facility. Once we established a partially lethal parasite inoculum and confirmed the feasibility of infrared BT measurement, we wished to evaluate the applicability of BT as a readily accessible and simple objective humane endpoint to predict lethal infections. Application of the 30 °C endpoint in a SMA survival study resulted in a ~30% mortality rate (Fig. 2a). Importantly, no unexpected fatalities occurred above the 30 °C threshold. This suggests that the endpoint was completely sensitive in capturing lethal SMA infections. However, not all animals deemed terminal by the threshold were deemed moribund by the researcher.

As measured by the infrared thermometer, the BT of all surviving animals dropped below 32 °C before recovering, reaching a low point of 30·8 ± 0·2 °C (Fig. 2b). Six surviving animals were recorded below 30 °C with the infrared thermometer but were ⩾30 °C with the rectal probe and proceeded to remain above 30 °C and recover. These measurements were all within a reasonable range of error between devices in all 6 animals (mean: 29·0 ± 0·3 °C). These data emphasize the importance of a rectal measurement for verification in this SMA model where a recovery phase is prevalent. These animals were not deemed terminally moribund.

Importantly, mice that reached a temperature below 30 °C were not all evaluated as terminally moribund based on the above-described clinical criteria.

https://doi.org/10.1017/S0031182014000821 Published online by Cambridge University Press

Due to ethical requirements, none of the mice were allowed to continue below 30 °C. Thus, the specificity of this endpoint could not be estimated.

Correlation analysis detected a weak correlation between BT and haemoglobin with a Spearman r-value of 0·62 [0·54–0·69]. When we separated animals into surviving and terminal groups based on BT <30 °C (Fig. 2c), haemoglobin concentration did not provide additional support for predicting lethality. For example, at day 10, when most animals became terminal, there was no difference in haemoglobin concentration (surviving: 1·7 (1·4, 1·9) mM *vs.* terminal: 1·4 (1·3, 1·5) mM). The correlation between BT and parasitaemia was low: Spearman $R = -0{\cdot}33$ [(−0·43)–(−0·22)], and did not provide additional support for predicting lethality (Fig. 2d).

DISCUSSION

Objectivity in the application of humane endpoints reduces user-bias and thus improves study designs in pre-clinical therapeutic trials, reliability in results and standardization between laboratories. Ultimately, the implementation of appropriate, objective humane endpoints reduces the number of animals required to test a given phenomenon.

Defining such an endpoint can be a challenge. It should be associated with disease progression and be predictive of lethality for use in survival investigations. Additionally, since survival is determined by a complex array of pathological dysfunction, it is difficult to define with a single measurement in a living animal. We have implemented BT measurements with success in the evaluation of ECM pathology. Our cut-off of 32 °C was determined based on a threshold past which erythropoietin-treated mice could not be saved (Wiese *et al*. 2008). In the years since implementation we have found this endpoint to be satisfactory for survival experiments. We have also benefitted from the simplicity of the measurement in training staff researchers compared with scoring of clinical symptoms (Lackner *et al*. 2006). Furthermore, BT has been used to follow disease progression in other infectious diseases in research of *Staphylococcus* and *Pseudomonas* (Soothill *et al*. 1992; Stiles *et al*. 1999), influenza (Wong *et al*. 1997) and fungal infections (Warn *et al*. 2003). Although weight loss is often used successfully as an objective humane endpoint in slow progressing disease research (Nemzek *et al*. 2004), we suggest that BT may be a better predictor of death in acute and progressive infectious disease research in rodents as it rapidly detects a dysfunction in maintenance of core BT: a complex and essential bodily function. Nevertheless, our experience with SMA infection as presented here is slightly more complicated due to the spontaneous recovery seen in this mouse model. It is intriguing that the SMA-mice sustain a BT approaching 30 °C (∼ 15% reduction in resting BT) and proceed to recover to a largely asymptomatic state. This recovery phase is a challenge for determining the specificity of the endpoint in this model. No unexpected deaths occurred with frequent surveillance (three times daily) and a BT limit of 30 °C; thus, the sensitivity for detecting lethality seems to be close to 100%. However, without pursuing a BT threshold below 30 °C, this endpoint may result in an over-estimation of the lethality. Indeed, in this study mice with an infrared and rectal BT below 30 °C were not all necessarily deemed terminally moribund. For the purposes of survival studies involving therapeutics, this over-estimation may be relevant. Thus, it seems that a composite endpoint involving a standardized clinical evaluation or investigation into a lower BT threshold may be appropriate in this model involving spontaneous recovery. Moreover, when surviving and terminal animals were defined with this endpoint haemoglobin levels were similar. Thus, lethality in this study may not be linked strictly to anaemia but be a complex result of malaria infection.

This predicament poses an ethical challenge when defining humane endpoints. It is of utmost importance to minimize the extent and duration of suffering in animal experimentation. This requires regular assessment of procedures and informed and well-functioning animal inspectorates/in-house veterinarians. In determining a robust predictor of lethality, a study involving potential predictors and utilizing death as the endpoint would be the most efficient. Moreover, the consequence of over-estimation of lethality may be an increase in false-negative hits concerning therapeutic efficacy, resulting in increased animal sample sizes and/or abandonment of the therapeutic (thus rendering the animal use redundant). However, as death is an unacceptable endpoint and ethical animal use requires that a study be powered to generate statistically useful data, a balance must be achieved based on the three *R*s of animal research (replacement, reduction and refinement) (Russell and Burch, 1959).

The major challenge with implementation of rectal BT measurements as a humane endpoint is the invasive nature of repeated rectal sampling. In disease models that progress rapidly from mild to severe presentation such as ECM, it is important to implement regular monitoring of pre-determined endpoints to capture terminal animals and reduce distress. Repeated rectal measuring is accompanied with an increased risk of intestinal perforation. This can lead to detrimental complications and distress, and confound the results generated.

In order to improve this measurement and ensure repeated monitoring of our endpoint, we considered substituting the rectal probe with a minimally invasive, non-contact infrared thermometer. This method is non-invasive but does require handling. In this study, we tested the performance of an infrared thermometer from one manufacturer. We suggest

https://doi.org/10.1017/S0031182014000821 Published online by Cambridge University Press

that pilot testing for optimization should be conducted for each thermometer and the emissivity index be determined for each strain of mouse.

As an alternative to infrared thermometry, temperature microchips that limit the handling of the animal are available but do require an invasive insertion of a foreign object, and may induce undesired inflammatory processes (Warn *et al*. 2003). The chip method may be more attractive for long-term investigations. A previous study with a similar infrared thermometry method in mice compared infrared and rectal thermometers and reported similar findings (Ochiai *et al*. 2007). A similar comparison was made between infrared and temperature microchips in mice with fungal infections where the mean difference between measurements techniques was negligible (Warn *et al*. 2003). However, in larger laboratory animals such as the rabbit and monkey it seems that dermal infrared thermometry does not strongly correlate with rectal measurements (Chen and White, 2006; Brunell, 2012). This may be due to reduced surface area to volume ratios in larger animals. In our study, despite high linear regression y-intercept values in malaria-infected mice, the difference between measurements varies about the zero value. This variation suggests that the rectal probe should be consulted when infrared measurement is $\pm 1{\cdot}0$ °C of the pre-determined endpoint. Our results support the replacement of the rectal probe for the implementation of BT as an objective humane endpoint in murine ECM and SMA pathology, where repeated measurements are required.

In conclusion we provide evidence that use of simple and affordable non-contact infrared emission technology can reliably improve surveillance frequency, reduce invasive monitoring and thus improve animal welfare in murine studies with acute neurovascular distress. In SMA, a temperature cut-off of 30 °C is successful in capturing all fatal infections and may be suitable as a humane endpoint for SMA although it may overestimate fatality. Investigation of a slightly lower BT cut-off may improve the specificity of the endpoint for SMA in mice.

ACKNOWLEDGEMENTS

We would like to acknowledge veterinarians Helle Nordahl Hansen, Grete Østergaard and Stefania Embla Arnorsdottir, and Casper Hempel and Flemming Fryd Johansen for productive discussions and/or observational assistance. We would also like to acknowledge the veterinarians at the Biocenter animal facility for expertise and continual care for animals.

FINANCIAL SUPPORT

This work was supported by the University of Copenhagen (BDV, JK, LM, FCC, JALK), Rigshospitalet (BDV, JK, LM, FCC, JALK), Novo Nordisk Fonden (BDV), Novo Nordisk (BDV) and Vifor Pharma (JK, LM, FCC).

REFERENCES

Brunell, M. K. (2012). Comparison of noncontact infrared thermometry and 3 commercial subcutaneous temperature transponding microchips with rectal thermometry in rhesus macaques (*Macaca mulatta*). *Journal of the American Association for Laboratory Animal Science* **51**, 479–484.

Chen, P. H. and White, C. E. (2006). Comparison of rectal, microchip transponder, and infrared thermometry techniques for obtaining body temperature in the laboratory rabbit (*Oryctolagus cuniculus*). *Journal of the American Association for Laboratory Animal Science* **45**, 57–63.

DellaValle, B., Staalsoe, T., Kurtzhals, J. A. and Hempel, C. (2013). Investigation of hydrogen sulfide gas as a treatment against *P. falciparum*, murine cerebral malaria, and the importance of thiolation state in the development of cerebral malaria. *PLOS ONE* **8**, e59271. doi: 10.1371/journal.pone.0059271.

Hein-Kristensen, L., Wiese, L., Kurtzhals, J. A. and Staalsoe, T. (2009). In-depth validation of acridine orange staining for flow cytometric parasite and reticulocyte enumeration in an experimental model using *Plasmodium berghei*. *Experimental Parasitology* **123**, 152–157. doi: 10.1016/j.exppara.2009.06.010.

Lackner, P., Beer, R., Heussler, V., Goebel, G., Rudzki, D., Helbok, R., Tannich, E. and Schmutzhard, E. (2006). Behavioural and histopathological alterations in mice with cerebral malaria. *Neuropathology and Applied Neurobiology* **32**, 177–188. doi: 10.1111/j.1365-2990.2006.00706.x.

Maretty, L., Sharp, R. E., Andersson, M. and Kurtzhals, J. A. (2012). Intravenous ferric carboxymaltose accelerates erythropoietic recovery from experimental malarial anemia. *Journal of Infectious Diseases* **205**, 1173–1177. doi: 10.1093/infdis/jis020.

Nemzek, J. A., Xiao, H. Y., Minard, A. E., Bolgos, G. L. and Remick, D. G. (2004). Humane endpoints in shock research. *Shock* **21**, 17–25. doi: 10.1097/01.shk.0000101667.49265.fd.

Ochiai, M., Yamamoto, A., Kataoka, M., Toyoizumi, H., Arakawa, Y. and Horiuchi, Y. (2007). Highly sensitive histamine-sensitization test for residual activity of pertussis toxin in acellular pertussis vaccine. *Biologicals: Journal of the International Association of Biological Standardization* **35**, 259–264. doi: 10.1016/j.biologicals.2007.01.004.

Russell, W. M. S. and Burch, R. L. (1959). *The Principles of Humane Experimental Technique*. Methuen, London, UK.

Soothill, J. S., Morton, D. B. and Ahmad, A. (1992). The HID50 (hypothermia-inducing dose 50): an alternative to the LD50 for measurement of bacterial virulence. *International Journal of Experimental Pathology* **73**, 95–98.

Stiles, B. G., Campbell, Y. G., Castle, R. M. and Grove, S. A. (1999). Correlation of temperature and toxicity in murine studies of staphylococcal enterotoxins and toxic shock syndrome toxin 1. *Infection and Immunity* **67**, 1521–1525.

Warn, P. A., Brampton, M. W., Sharp, A., Morrissey, G., Steel, N., Denning, D. W. and Priest, T. (2003). Infrared body temperature measurement of mice as an early predictor of death in experimental fungal infections. *Laboratory Animals* **37**, 126–131. doi: 10.1258/00236770360563769.

Wiese, L., Hempel, C., Penkowa, M., Kirkby, N. and Kurtzhals, J. A. (2008). Recombinant human erythropoietin increases survival and reduces neuronal apoptosis in a murine model of cerebral malaria. *Malaria Journal* **7**, 3. doi: 10.1186/1475-2875-7-3.

Wong, J. P., Saravolac, E. G., Clement, J. G. and Nagata, L. P. (1997). Development of a murine hypothermia model for study of respiratory tract influenza virus infection. *Laboratory Animal Science* **47**, 143–147.

Yap, G. S. and Stevenson, M. M. (1992). *Plasmodium chabaudi* AS: erythropoietic responses during infection in resistant and susceptible mice. *Experimental Parasitology* **75**, 340–352.

APPENDIX

Measurements of body temperature by rectal probe and infrared thermometers from Fig. 1 are plotted as the difference between rectal probe and thermometer in matched measurements. Measurements were taken after cervical dislocation (CD), cerebral malaria (ECM) and severe malarial anaemia (SMA).

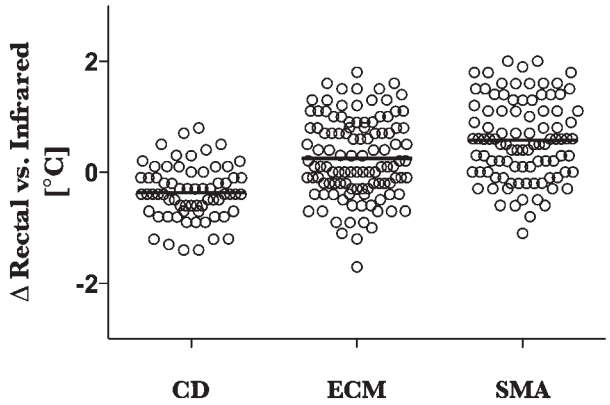

Fig. A1. Difference in rectal probe versus infrared in measuring body temperature in C57BL/6J and A/J mice.

https://doi.org/10.1017/S0031182014000821 Published online by Cambridge University Press