To all registered users of the '*P. berghei* SharePoint Site' (Leiden Malaria Research Group).

We would like to share some observations on different *P. berghei* isolates, stabilates and laboratory lines.
These observations may be of relevance for studies on host-parasite interactions and on severe disease states exhibited by mice such as experimental cerebral malaria (ECM) or lung pathology. Moreover, they have implications for analyzing and reporting of 'growth phenotypes' of blood stages of genetically modified *P. berghei* mutants.

We also provide this information with the aim of obtaining a better insight into the genotype and phenotype of the different parasite lines used and described by different laboratories. This knowledge may lead to a better standardization of studies using the rodent model *P. berghei*, as was mentioned in a recent consultation report/paper discussing the role of animal models for research on severe malaria (Craig et al.; 2012; PloS Pathogens)

Below we summarize a number of (historic and recent) observations on the (genetic analyses of) different *P. berghei* isolates/ stabilates/laboratory lines. This information has been collated after discussion and in collaboration with Oliver Billker, Matt Berriman (Sanger Institute, UK); Sarah Reece, Ricardo Ramiro (Edinburgh University, UK); Philippe van der Steen (Catholic University of Leuven, Belgium); and Annette Erhart (Institute of Tropical Medicine Antwerp, Belgium).

Most of the information provided below and additional information on different *P. berghei* isolates/laboratory lines is available on the (password-protected) Leiden SharePoint site (folder 'Shared Documents'; subfolder *P. berghei* isolates and lines). We very much appreciate additional comments and information and this additional information would be welcomed on the Leiden SharePoint site.

Naming of different *P. berghei* isolates and stabilates available from the Edinburgh collection
To our knowledge only the Edinburgh University has a collection of (stabilates of the) original *P. berghei* isolates that are well described with respect to the history of the stabilates. These stabilates are not available from MR4 (www.mr4.org).
See the description of the 6 original *P. berghei* isolates: ANKA, K173 (N-line, RC line), KSP11, LUKA, NK65, SP11 (line RLL) and the description of the various *P. berghei* stabilates of these isolates, present in the Edinburgh Collection.

Some characteristics of the Edinburgh *P. berghei* isolates

- The iso-enzyme composition (iso-enzyme variants of GPI, 6PGD, LDH, GDH) is the same for all *P. berghei* isolates (Beale, G.H., Carter, C and D. Walliker (1978) Genetics. In: Rodent Malaria (R. Killick-Kendrick and W. Peters, eds.).
- Only 'small' differences in karyotypes (size of chromosomes, chromosomal location of genes) have been found between parasites of the Edinburgh *P. berghei* stabilates (more detailed information on the karyotypes is available from the Leiden SharePoint site).
- Recent analyses of gametocyte production of the Edinburgh stabilates indicate that only parasites of the Edinburgh ANKA and NK65 stabilates produce gametocytes. No gametocytes were observed in stabilates of other isolates (Leiden analyses; unpublished)
- Recent analyses of blood infections in mice of the Edinburgh lines indicate that only parasites of the Edinburgh ANKA isolate show a clear CD36-mediated schizont sequestration phenotype as described by Franke-Fayard *et al*. (2010; PloS Pathogens). In all other lines schizonts were observed in the peripheral blood circulation (tail blood), both at low and high parasitemias (Leiden analyses; unpublished)

Low genetic diversity between the different *P. berghei* isolates or 'contamination' of the stabilates?

Sequencing of a selected number of genes of the Edinburgh isolates has provided evidence that all these parasites, except K173 (RC) and KSP11 (RLL), may be genetically identical since they all have identical gene sequences for *ama1, msp1* and *dhfr.* Based on these observations it has been suggested that there may be a cross-contamination that has occurred among the Edinburgh stabilates (see Saul, A. et al., 1997, Mol. Biochem. Parasitol 84, 143 – 147). Whole-genome sequencing efforts at Sanger of different *P. berghei* lines from different origins indicate however that sequence diversity in *P. berghei* is indeed minimal (see below).

***P. berghei* ANKA, NK65, K173 laboratory lines that are used in other laboratories**

In different laboratories mainly parasites of the ANKA, NK65 and K173 isolates are used, however the origin and history of these 'laboratory lines' is less well described than the Edinburgh isolates and stabilates.

ANKA: Different lines that originate from the ANKA isolate are used. ANKA reference lines from the Leiden group are all generated in a cloned line using a *P. berghei* stabilate of ANKA, which was stored at the Institute of Tropical Medicine Antwerp soon after its isolation from *A. dureni* (Prof Marc Wery; Wery M., et al.,; 1979; Ann. Soc. Belge Med. Trop. 59:347–360). This cloned line, *P. berghei* ANKA*wt* (cl15cy1) has been used at the Sanger Institute to generate the available reference genome sequence. It produces gametocytes, induces ECM in ECM-sensitive mice, schizonts show a CD36-mediated sequestration phenotype and it can complete the whole lifecycle including development in mosquitoes and in the liver. Most fluorescent and luminescent transgenic reporter *P. berghei* lines generated by the Leiden group are made in this background parasite line.

NK65: Different laboratories use NK65 parasite lines that originate from NK65 parasites maintained and propagated in New York (see Vanderberg, J.P. et al., (1968); J. Parasitol. 54: 1009-1016). This NK65 'New York' line produces gametocytes. The NK65 New York parasite lines are frequently used for studies on liver stage infection. Other laboratories use NK65 parasites, directly derived from the Edinburgh collection (NK65 'Edinburgh'), which also produces gametocytes. For example NK65 Edinburgh has been used to develop a model for malaria-associated acute respiratory distress syndrome (MA-ARDS; van den Steen et al., 2010; Am J Respir Crit Care Med. 2010, 181(9):957-68). We were unable to find detailed information on the history of the NK65 New York lines that are now used in different laboratories. Since parasites of different NK65 lines do not induce ECM in C57BL/6 mice, these parasites are often used as 'control parasites' in studies on ECM in *P. berghei* ANKA infections.
K173 parasites have been used in Nijmegen (The Netherlands) for studies on ECM in C57BL/6 mice (Curfs et al, 1990. J. Exp. Med. and other papers). This laboratory line, which has been maintained for many years by mechanical passage in mice, does not produce gametocytes and schizonts do not sequester (Franke-Fayard et al, 2010; PloS Pathogens). Interestingly, K173 parasites that have been used in different laboratories in Australia do NOT induce ECM in CBA mice (Sanni et al, 2001, Am J Pathol and other papers). The relationship/history of the K173 Nijmegen and K173 Australia lines is unclear. The karyotype of the Nijmegen K173 parasites has been analysed and is clearly distinct from ANKA and NK65 parasites (please click here for information on karyotypes of K173 Nijmegen and ANKA Leiden lines). To our knowledge no genotype information is available for the K173 Australia parasites.

Other *P. berghei* isolates

Other *P. berghei* isolates/lines (LUKA, KSP11, SP11) are not frequently used and it is unclear whether other institutes (other than the University of Edinburgh) have stabilates of parasites of the 'original' LUKA, KSP11, SP11 and K173 isolates that still produce gametocytes.
In Leiden we have recently analysed parasites of K173, SP11, SP11 (RLL line) stabilates from stocks available at the Institute of Tropical Medicine Antwerp. We found evidence for gametocyte production in SP11 and SP11 (RLL). We have obtained cloned lines from SP11 (SP11 Antwerp clones) that produce gametocytes and schizonts show a schizont-sequestration phenotype comparable to ANKA parasites. Preliminary karyotype analyses of SP11 parasites indicate that the karyotype of these parasites is highly similar to that of *P. berghei* ANKA (and NK65) parasites (Leiden analyses, unpublished).

Genotyping of the different isolates/lines

Only limited information is available for the genotype (and differences in genotype) of the different *P. berghei* isolates and laboratory lines. To our knowledge no diagnostic PCR or other assays have been described for genotyping the different isolates and laboratory lines. Detailed karyotype comparisons are limited. For ANKA lines/clones it has been shown that chromosomal rearrangements occur frequently during asexual propagation, resulting in significant size

differences between homologous chromosomes. It is therefore uncertain whether chromosome karyotyping can be used as a diagnostic characteristic to differentiate between parasites of different isolates. For example, parasites of the ANKA, NK65 and SP11 parasites show relatively similar karyotypes (Leiden, unpublished analyses). On the other hand, the ANKA Leiden and K173 Nijmegen show clearly distinct karyotypes and these differences appear to be 'relatively' stable.

Efforts are ongoing at Sanger to sequence the genome of parasites of the following lines i) SP11 Antwerp clone 1 (SP11 parasites obtained from Annette Erhart, Antwerp; cloned in Leiden); ii) SP11 (RLL) uncloned (parasites obtained from Annette Erhart, Antwerp); iii) NK65 New York (obtained from Robert Menard, Paris) and iv) NK65 Edinburgh (obtained from Philippe van den Steen, Leuven and cloned in Leiden, 1995cl1) v) K173cl1 (K173 parasites obtained from Nijmegen; cloned in Leiden). Preliminary analyses of the sequence data show very limited sequence variation between ANKA Leiden, NK65 New York, NK65 Edinburg and SP11 Antwerp. More variation is found in K173 Nijmegen and SP11 RLL Antwerp. Parasites of both K173 Nijmegen and SP11 RLL Antwerp have been maintained for long(er) periods by mechanical transmission in mice and it is unknown whether the higher sequence diversity is present in the original isolates or is an 'artifact' of mechanical transmission.

***P. berghei* isolates and laboratory lines: ECM properties**

- Most ANKA lines used in different laboratories induce ECM in C57Bl/6 or CBA mice (and in most, but not all outbred MF1 and Swiss mice)
- K173 Nijmegen parasites induce ECM in C57Bl/6 (also see below)
- K173 Australia parasites do not induce ECM in CBA mice.
- NK65 New York parasites do not induce ECM in C57Bl/6 (?)
- NK65 Edinburgh parasites do not induce ECM in C57Bl/6

To our knowledge, it is unknown if LUKA, KSP11 or SP11 induce ECM in C57Bl/6 or CBA mice

Is the ability to induce severe disease (ECM, MA-ARDS) a stable feature of the different *P. berghei* isolates or laboratory lines?

- Evidence has been presented that cloned lines of *P. berghei* ANKA can differ in the ability to induce ECM (Amani et al., 1998; Infect. Immunn. 66, 4093-9)
- Evidence has been presented that host diet can influence the capacity of *P. berghei* lines to induce ECM (Levander et al, 1995; J Parasitol 81, 99-103)
- In Leiden we have a cloned line from the Nijmegen K173 (Nijmegen K173cl1) which 'has lost' the capacity to induce ECM in C57BL/6 mice
- In Leiden we have observed differences in the ability to induce ECM in C57Bl/6 mice between different experiments when using *P. berghei* ANKA clones (see below)
- NK65 Edinburgh and NK65 New York show differences in their ability to induce MA-ARDS (Leuven analyses, unpublished)

Are other blood stage growth characteristics stable features of different *P. berghei* isolates or laboratory lines?

Not many features of blood stage growth of the different *P. berghei* isolates/laboratory lines have been analysed in a quantitative manner. However, in several more recent studies blood stage growth has been compared between genetically modified mutants and their 'wild type' parent parasites, mainly using ANKA parasites. Blood stage growth has been determined by two methods 1) measuring parasitemia after intravenous inoculation of a single parasite during a cloning procedure or 2) measuring parasitemia after intraperitonal or intravenous injection of 100-10.000 parasites

In Leiden we have analysed the growth of >1500 clones of *P. berghei* ANKA using method 1. Parasitemia is measured on day 7-10 after inoculation of a single parasite till a parasitemia of 0.5-2% had been reached (in Swiss mice; 25g). More than 95% of 'wild type' ANKA clones show a parasitemia of 0.5-2% at day 8 which results from a (very stable) multiplication rate of 10X per 24 hour (Spaccapelo et al, Am J Pathol, 2010, 176, 205-17). Growth rates of wild type parasites in mice with parasitemias >2% are less predictable as there is a much larger variation in multiplication rate. This variation is influenced by a combination of factors including but not restricted to differences in i) 'preference for invading reticulocytes' (see below), ii) percentage of multiply infected rbc and iii) timing and production of reticulocytes by the host.

Growth and reticulocyte preference of different *P. berghei* isolates or laboratory lines

P. berghei parasites have a strong preference for invading reticulocytes. We are not aware of detailed studies which have compared differences in reticulocyte preference/restriction between different *P. berghei* isolates or laboratory lines. In Leiden we have made the following observations:

- Most *P. berghei* ANKA lines have a strong preference for invasion into reticulocytes. During the course of an infection, when the availability of reticulocytes becomes a limiting factor, most ANKA parasite lines have the capacity to 'switch to invading normocytes'. When reticulocytes are available again in the circulation, parasites 'switch back' to invading reticulocytes
- In different hosts *P. berghei* ANKA clones show differences in the capacity to switch to invading normocytes'. For example, in WISTAR rats the switch to normocyte invasion is (nearly) absent.
- In general parasites of NK65 lines have a stronger preference for reticulocyte invasion in comparison with parasites of ANKA lines
- Between experiments, or in different mice, parasites of the same ANKA clone can either switch to normocyte invasion or remain restricted to invasion of reticulocytes. Infections in mice then have two typical courses of parasitemia. Initially all infections have a typical reticulocyte restricted course of infection until parasitemias reach 0.5-2%; mice infected with parasite that make the switch to invading normocytes than rapidly increase in parasitemia from 0.5-2% to 15-25% within 2 days (at which stage most mice will succumb to ECM in ECM-sensitive mice). In other infections (mice) parasites remain reticulocyte restricted and in these infections there is an actual small drop in parasitemia at around 3-5% parasitemia as consequence of a shortage of

reticulocytes in circulation. After this short second phase of a reduced multiplication the parasitemia then again rapidly increase a result of strong 'a wave' of reticulocyte production. In these infections mice usually do not die of ECM but eventually die from a fulminating parasitemia. This second course of infection is much less predictable as a result of different factors as mentioned above. Outbred Swiss mice that do not develop ECM usually follow a 'reticulocyte-restricted' course of parasitemia, whereas Swiss mice that do die from ECM make the switch to invading normocytes.

- We have found evidence that differences exist in the capacity to switch to invading normocytes between different ANKA clones. Whether these differences are 'genetically fixed' in these clones or are 'reversible' is largely unknown due to the lack of detailed analyses of (host and parasite) differences in multiple experiments and the relatively large inter-experimental variation in the capacity to switch to invading normocytes.
- The Leiden K173cl1 is 'restricted' to invasion of reticulocytes and does not switch to invading normocytes. This clone was obtained from the K173 Nijmegen line that invades both reticulocytes and normocytes. This clone does not induce ECM in C57Bl/6 mice.

Concluding remarks

As a consequence of the above described observations of inter-clonal and inter-experimental variation, we believe that standardization between experiments is vital for analysing genetically modified *P. berghei* mutants and for linking specific modifications to growth- and virulence phenotypes. In order to achieve such standardization the following aspects should be considered for analysis and reporting mutant phenotypes: (i) providing information on the origin (and genotype) of the parent *P. berghei* line that has been used for genetic modification; (ii) details of the host strain used (iii) determination of growth- and virulence characteristics in standardized assays, and where possible (vi) analysis of growth/virulence characteristics of two mutants that are derived from two independent transfection experiments or analysis of restoration of the wild-type genotype and phenotype in complementation experiments.

We hope you find what we have written of some use and, as we mentioned above we very much appreciate any comments, remarks or information you may have (see further below).

With respect to our discussions about sequencing *P. berghei* isolates/strains in addition to the ANKA sequences I have tried to summarize a number of (historic and recent) observations on the (genetic analyses of) different *P. berghei* isolates/ strains/laboratory lines which may be of use

David Walliker collected and described the following 'isolates' that are available in Edinburgh: K173 (N) (1948, G. surdaster; this strain is 'the type' P. berghei species), ANKA (1965/1966, A dureni), KSP11 (1961, A. dureni), LUKA (1966 A dureni), NK65 (1964, A dureni), SP11 (1961, A dureni). See the attached PDF-document for further information. These lines are still available in Edinburgh and have recently been shipped also to Leiden (and Glasgow?).

- Sequencing of a few genes of the Edinburgh isolates has 'provided evidence that all these parasites, except K173 (RC) and KSP11 (RLL), may be genetically identical since they all have identical gene sequences for *ama1, msp1* and *dhfr* indicating possibly some cross-contamination among laboratory lines of *Plasmodium berghei* (see Saul, A., Prescott, N., Smith, F., Cheng, Q. and Walliker, D. (1997) Molecular and Biochemical Parasitology 84, 143 – 147).

- Ricardo Ramiro (from Sarah Reece's group, Edinburgh) has sequenced the *p47, p48* and (the fast evolving region of) *p230* genes of a number of these isolates and he found no sequence diversity

- The iso-enzyme composition (iso-enzyme variants of GPI, 6PGD, LDH, GDH) is the same for all *P. berghei* isolates (Beale, G.H., Carter, C and D. Walliker (1978) Genetics. In: Rodent Malaria (R. Killick-Kendrick and W. Peters, eds.) pp 1-52. Academic Press, London).

- In Leiden we recently checked gametocyte production (and reticulocyte preference and schizont sequestration) of the different Edingburgh isolates. **For only the ANKA and NK65 isolate we could detect gametocyte production**!

- In Leiden we analysed FIGE-karyotypes (chromosome size) of the Edinburgh isolates. Due to variability of the subtelomeric regions of chromosomes, the karyotype analyses (see PowerPoint attachment) did not provide much additional insight in the (dis)similarity of the genotypes of the different isolates. (Small) differences in the sizes of individual chromosomes between isolates can also be observed between clones of a single isolate. These size differences are mainly the result of the loss (or acquisition) of subtelomeric repeat sequences. Most isolates showed '*P. berghei*-like' karyotypes that were relatively comparable and were similar to published ANKA/K173 karyotypes. KSP11 showed a translocation of one of the *rrna* gene units to a different chromosome. **The karyotype of the 'Leiden/Nijmegen K173 clone' (K173cl1) that has been sequenced by Sanger shows a very similar karyotype to the K173 (N) Edinburgh isolate (see also below).**

- Oliver Billker has provided some gene comparison sequence data of different *P. berghei* 'isolates' available in in GenBank (see attached excel file). Since the exact history/origin of the parasites is often not known and different laboratories work with different 'laboratory lines' of the original isolates, it may be better to name these 'laboratory lines originating from the …. isolates' (see also below). These comparisons indicate a larger sequence diversity between the genes of 'laboratory lines from different isolates' than expected based on the sequence information available from the Edinburgh isolates. The question arises whether the reported sequence diversity has arisen during maintenance of the parasites in the laboratory or if it already existed between the isolates.

- Sanger has sequenced the genome of parasites of the Leiden/Nijmegen K173 (K173cl1) line. DNA was provided by Blandine Franke-Fayard and Chris Janse. This line does not produce gametocytes and schizonts do not sequester. The FIGE karyotype is somewhat different from that of the ANKA line; a number of chromosomes show a reduced size mainly as a result of the loss of the subtelomeric 2.3kb repeats (see below for more details about the phenotype and history/origin of this line). Thomas Otto has produced overviews on SNPs and indels in all genes in a comparison of ANKA and K173. Of the ~5000 genes, ~4000 showed no sequence diversity!! (Including P48, P47, P230); ~850 genes showed (a low number of) SNPs and/or indels; less than 50 genes showed deletions.

- What is known of (defined) phenotypic differences between *P. berghei* isolates or laboratory lines?

Gametocyte production: Evidence for gametocyte production has only been found for parasites from the ANKA and NK65 isolate (see above). It appears that the other lines 'have lost' gametocyte production during laboratory passage in mice (or due to contamination of the different isolates; see Saul A., 1997, Mol Biochem Parasitol). The Leiden K173 line does not produce gametocytes (K173cl1, origin and history of this line is not clear; this line was obtained from Nijmegen where they have used it since the 60ties for studies on cerebral complications and has been maintained for > 10 years by mechanical passage in mice, see also below). Gametocyte production can rapidly be lost during mechanical passage in mice, even in cloned lines from the ANKA and NK65 isolates, thus it is not a stable feature of lines.

Schizont sequestration: Schizonts of the ANKA strain show a distinct sequestration phenotype. We have some evidence that schizonts of LUKA and KSP11 show a less clear 'schizont sequestration phenotype' compared to ANKA schizonts. Schizonts of Leiden/Nijmegen K173 do not sequester

Experimental cerebral complications in C57Bl6 mice (ECM): Infections with ANKA but not NK65 results in ECM. K173 parasites used by Nijmegen induce ECM (Curfs et al, 1990. J Exp Med and

other papers) but K173 parasites used in Australia do not induce ECM (Sanni et al, 2001, Am J Pathol and other papers). The relationship/history of the K173 Nijmegen and K173 Australia is not clear. Interestingly, the sequenced Leiden K713cl1 line that has been derived from the K173 Nijmegen line shows reduced ECM (unpublished observations Chris Janse, Blandine Franke-Fayard). We believe no information on ECM is available from the other isolates (?). We have evidence that the capacity to induce ECM can decrease or can even be lost from laboratory lines during mechanical passage in mice. It seems that ECM is also not a very stable feature of isolates/lines. In the Leiden lab we have observed a correlation between a restriction to 'reticulocyte- invasion' and ECM (see below).
Reticulocyte preference: All *P. berghei* parasites show a strong preference for reticulocytes invasion (and growth). Therefore problems with long term *in vitro* culture of *P. berghei* is a consequence of rodent reticulocytes maturing rapidly in culture and P. berghei in normocytes show a reduced ability to fully mature. However, between different isolates and laboratory lines differences exist in the capacity of invading normocytes. NK65 parasites are more restricted to invading reticulocytes than ANKA. ANKA (and K73 Nijmegen) parasites have to capacity to (switch to) normocytes invasion, which usually occurs when parasitemias rises above 1-3%. However, in Leiden we have cloned lines of ANKA and K173 that show a reduced ability to (switch to) normocyte invasion. Most lines that show a reduced invasion of normocytes, also show reduced ECM (day 6-9 after infection) and most mice die from severe disease/anemia in week 2/3 after infection

Lung pathology: Infections in C57CBL6mice with parasites of the Edinburgh NK65 strain show features of malaria-associated acute respiratory distress syndrome (MA-ARDS; week 2-3 after infection) as shown by Philippe van der Steen at the Leuven University (Van den Steen et al, 2010, Am J Respir Crit Care Med). Philippe recently found that a NK65 line obtained from Leiden showed a different lung pathology (and course of parasitemia) compared to the Edinburgh NK65 line. This NK65 line is a GFP-Luciferase expressing reporter line made in a NK65 line that has been obtained from Robert Menard (Paris) that originates from a 'New York' NK65 line. The history and the relationship between the NK65 (New York) and NK65 (Edinburgh) line is not completely clear. In Leiden we have recently compared the FIGE-karyotype of both NK65 lines and observed several differences in chromosome patterns (see attached PowerPoint file).

Because of the inherently unstable phenotypic features -gametocyte production, ECM, lung pathology, normocyte invasion of laboratory lines of the different isolates- it might be questionable whether sequencing of other **isolates** will provide additional insight in processes underlying these features. In addition, the lack of gametocyte production and the limited sequence difference between the isolates in Edinburgh may question the value of additional sequencing of Edinburgh isolates.
At this moment the best choice for sequencing would be in our view the NK65 line (New York or Edinburgh?) because of its capacity to produce gametocytes.