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Infection dynamics of *Cryptosporidium parvum* in ICR outbred suckling mice

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Abstract. An ICR outbred suckling mouse model of cryptosporidiosis was used to explain some of the variability associated with experimental *Cryptosporidium parvum* infections in neonate mice. Fourty four groups of 12 mice each, ranging in age from 4–12 days, each received 1.0×10^4 CsCl purified oocysts *per os* in 5 μm PBS. At 6 days post-inoculation (PI), mice were killed by CO_2 overdose and individually weighed. Intestines were then homogenized and oocysts were quantified by hemacytometer. Results revealed that both age and weight have pronounced effects on numbers of oocysts produced *in vivo*, with larger and older mice producing higher numbers of parasites. Mice 8–9 days of age at the time of inoculation displayed the least amount of weight dependent variability, produced the highest numbers of oocysts, and were judged to be superior over other ages for pharmaceutical screening. Significant reductions in numbers of oocysts occurred in mice inoculated at 10 days of age, and only a few oocysts were found in mice inoculated at 11–12 days of age. These studies suggest that at least some data on *Cryptosporidium* generated from suckling mouse studies to date are probably unreliable and should be viewed skeptically.

Cryptosporidium parvum Tyzzer, 1912 is an apicomplexan that infects the intestinal epithelial cells of numerous mammals, including humans (Fayer et al. 1990, Current and Garcia 1991, Sterling and Arrowood 1992). This parasite is thought to infect 10–20 % of all AIDS patients with diarrhea in North America and up to 55 % of AIDS patients in some Third World countries (Chui and Owen 1994). Currently, no effective therapy exists for treating the disease in humans at non-toxic levels (Moore et al. 1988, Fayer et al. 1990, Rehg 1994a).

A wide variety of animal models have been developed to study cryptosporidiosis *in vivo*. These include immunosuppressed rat (Rehg et al. 1987, 1988a,b, Brasseur et al. 1988, 1991, 1993, 1994, Rehg and Hancock 1990, Rasmussen et al. 1991, Rehg 1991a,b,c, 1993, 1994b, 1995, Leméteil et al. 1993, Verdon et al. 1994, Roussel et al. 1995), athymic (nude) mouse (Heine et al. 1984, Ungar et al. 1990, Bjorneby et al. 1991, Mead et al. 1991, Perryman and Bjorneby 1991, Kwa et al. 1993, Leitch and He 1994), neonatal, aged or immunosuppressed hamster (Kim 1987a,b, Rossi et al. 1990, Rasmussen and Healey, 1992a,b), SCID mouse (Mead et al. 1991, 1995, Kuhls et al. 1992, Chen et al. 1993a,b, Perryman et al. 1993, Cama et al. 1994, Petry et al. 1995), immunosuppressed adult C57BL/6N or ICR mouse (Kimata et al. 1991, Rasmussen and Healey 1992c, Yang and Healey 1994, Rasmussen et al. 1995), and the various neonatal (suckling) mouse (Tzipori et al. 1982, Ernest et al. 1986, Blagburn et al. 1991, Enriquez and Sterling 1991, Novak and Sterling 1991, Tilley et al. 1991a,b, Fayer and Ellis, 1993, 1994, Finch et al. 1993, Koudela and Heřmánek 1993, Burad et al. 1995) models. Although all have varying degrees of usefullness, each of these models suffers from one or more problems. These include cost, amount of labour involved in parasite quantitation, necessity of a pathogen-free environment for the immunosuppressed models, and high degree variability both among animals and between experiments.

We were interested in developing an *in vivo* model that was relatively cost effective, simple, reproducible, and applicable to large numbers of animals per group. Once designed, this model could be used to test pharmaceuticals following *in vitro* screening using the recently developed 96-well ELISA (Woods et al. 1995). We chose to concentrate on an ICR outbred suckling mouse assay as these mice are the least expensive to assay per animal and entire litters can be treated with a pharmaceutical, which allows for a large N per dose regime of compound. In addition, ICR outbred mice breed easily, produce high numbers of offspring, and the females tolerate manipulation well and readily accept offspring from other dams, which permits mixing and matching among litters. We also wanted to develop a rapid quantitation method without the use of histology, and the suckling mouse intestine was the only model devoid of intestinal particulate debris, which is important if oocysts are to be quantified accurately and without using some form of arbitrary scoring.

MATERIALS AND METHODS

Oocysts of the KSU-1 isolate of *Cryptosporidium parvum* were purified from the feces of 5 day old calves (*Bos taurus* L.) by sucrose and CsCl gradient centrifugations as described previously (Taghi-Kilani and Sekla 1987, Upton et al. 1994). Oocysts were 2–5 weeks of age at the time of inoculations. All procedures involving calves were approved by the Institutional Animal Care and Use Committee.

Late gestation, ICR outbred female mice (*Mus musculus* L.) were purchased commercially (Harlan Sprague Dawley, Indianapolis, IN). Females were placed individually in plastic cages with stainless steel lids and provided wood shavings for bedding. Commercial rodent pellets and water were supplied *ad libitum*. Cages were examined for litters at 12 h intervals. On the day of inoculation, which represented a neonate age of 4–12 days, litters ≥12 were adjusted in size to 12 by removing the smallest extra mice, and each mouse was inoculated orally with 1.0×10^4 CsCl purified oocysts in 5 µl PBS by micropipette. A maximum of two groups of mice representing a single age were inoculated on any given day. All mice were killed by CO_2 overdose (Andrews et al. 1993) at 144 h (6 days PI) and the entire intestinal tract (both small and large intestines, but not stomach) of each mouse removed. Intestines were individually homogenized in 2 ml PBS using 6 strokes of a motor-driven teflon-coated tissue grinder. The final volume of the homogenate was brought up to 10 ml with an aqueous solution of 2.5 % (w/v) $K_2Cr_2O_7$. The number of oocysts produced per mouse was quantitated using a hemacytometer. All procedures involving mice were approved by the Institution Animal Care and Use Committee.

RESULTS AND DISCUSSION

Figure 1A represents a scatter plot where mouse age at the time of inoculation, mean numbers of oocysts (± SD) produced per litter, and mean litter weight at necropsy are compared. Figure 1B represents a 3-dimensional view of this data when standard deviations are omitted. These results show that a minimum of two variables affect oocyst production in ICR suckling mice. The first is size; larger mice of the same age tend to produce higher numbers of oocysts than smaller, age-matched groups (for example, compare the four groups of seven day old mice among themselves). Second, suckling mice inoculated when very young or very old tend to develop poor infections (for instance, 4, 11, and 12 days old mice) whereas mice inoculated at 5–9 days of age tend to develop good infections. At 10 days of age, a significant drop occurs in oocyst numbers, which appears to be correlated with final mean litter weight; litters with larger suckling mice tend to produce fewer oocysts than do litters with smaller animals. These results show clearly that both age and weight of a suckling mouse have a significant impact on oocyst production.

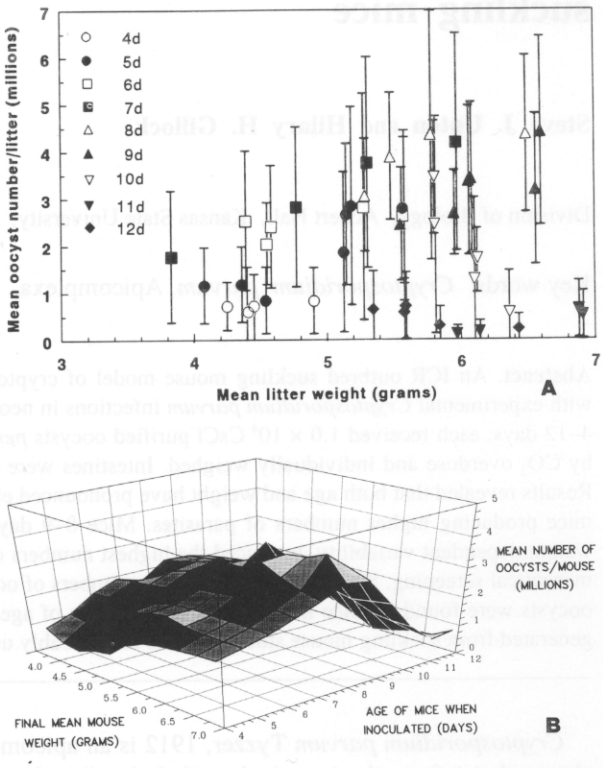

Fig. 1. Effects of age and weight on numbers of oocysts produced 6-days post-inoculation in ICR outbred suckling mice. **A** – scatter plot showing mean numbers of oocysts/litter (± SD) plotted against mean of final litter weight for mice inoculated 4–12 days of age (see symbol legend in figure). **B** – 3-dimensional graph of data from 1A.

Figure 2 represents a breakdown of the data in Figure 1 where both numbers of oocysts and weight for each mouse are plotted as a ratio. The x-axis represents the weight of individual mice divided by the mean litter weight at necropsy. A ratio < 1.00 means that the mouse is smaller than the mean litter weight, whereas a mouse > 1.00 is larger. The y-axis represents the same type of ratio, only the numbers of oocysts/mouse were divided by the mean numbers of oocysts/litter. Thus, ratios < 1.00 represent mice producing fewer oocysts than the mean whereas ratios > 1.00 are high oocyst producers. These ratios allow differences such as quality of parental care, differences in oocyst age and viability at the time of inoculation, and minor differences in oocyst quantitation of the inoculum to be negated. Results show that neonates 4–7 days of age at the time of inoculation produce oocysts in a size dependent manner, with larger mice tending to produce higher numbers of oocysts than runts. However, the weight related effect tends to level out when mice are inoculated at 8 and 9

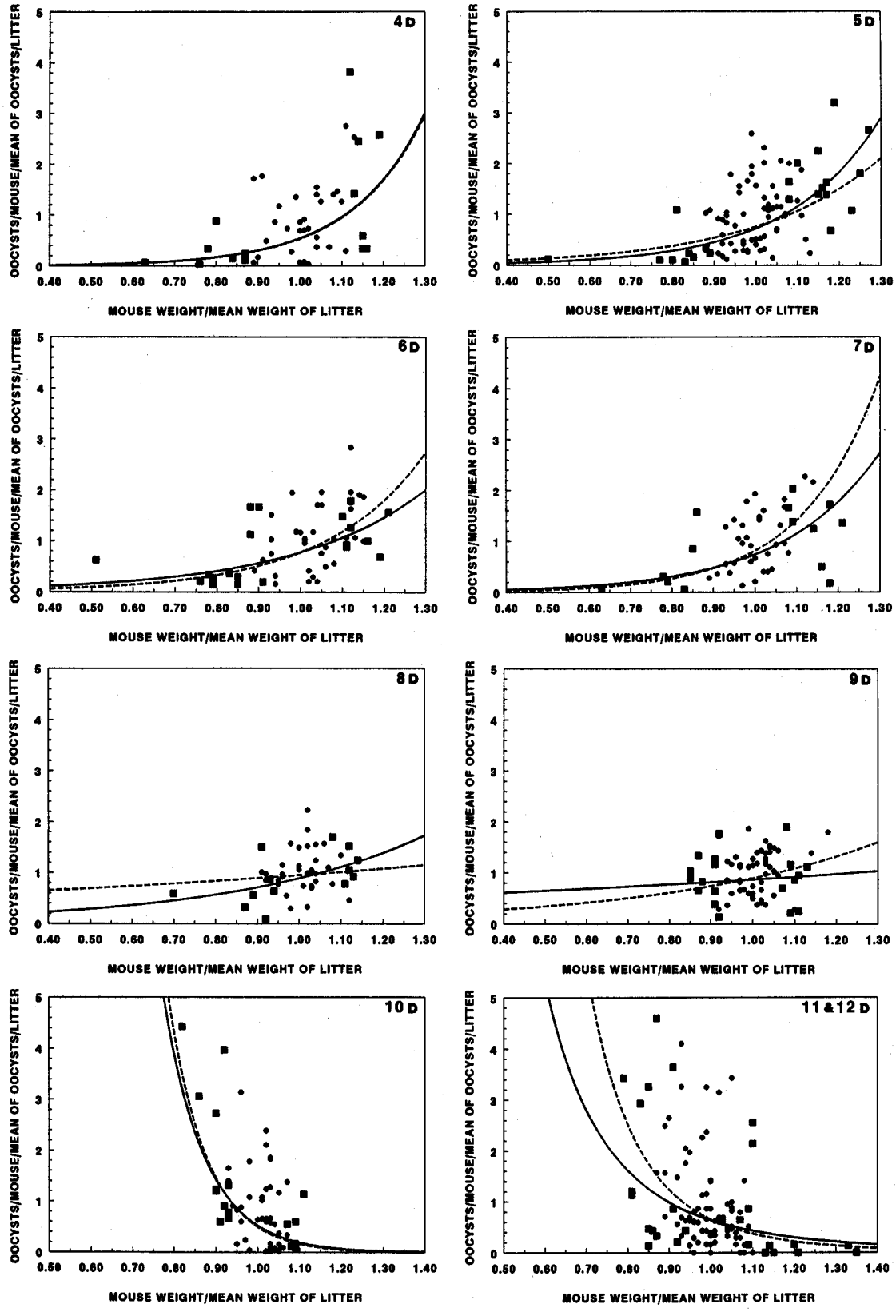

Fig. 2. Scatter plots of data in Fig. 1 for each mouse in each age group. Data was converted into a ratio where the x-axis is the weight of a mouse at necropsy divided by the mean litter weight, and the y-axis the numbers of oocysts/mouse divided by the mean numbers of oocysts/litter. The solid line represents a computer generated curve based on data points from all animals whereas the dashed line is a curve when animals with weights outside the 95 % confidence limits are excluded.

days of age, suggesting these age groups may be optimal for drug-related studies. At 10 days of age, a reversal in the trend is noted, with lighter mice clearly producing higher numbers than heavier animals. A final weight > 6 grams at the time of necropsy indicates a waneing infection for mice inoculated at 10 days of age. Solid lines represent computer generated curves of results from all mice (solid circles and squares) and dashed lines represent only those mice with weights lying within the 95 % confidence level (solid circles only).

Previous studies have shown that the intensity of *Cryptosporidium parvum* infections in murine models is affected by mouse strain (Enriquez and Sterling 1991, Rasmussen and Healey 1992c). However, the mechanisms by which age and weight affect oocyst production are not yet understood, although the age related susceptibility to infection has been noted (Mead et al. 1991, Novak and Sterling 1991, Kuhls et al. 1992). It is possible that embryonic and developmental antigens in the intestine play a role as significant changes in the glycoconjugate composition of the rodent gut occur during development (Shub et al. 1983, Torres-Pinedo and Mahmood 1984, Srivastava et al. 1987, Ozaki et al. 1989, Taatjes and Roth 1990).

Currently, most infections in rodents are quantified using one of three methods, but all have some degree of inherent variability. The most widely used method, histology, was developed by Riggs and Perryman (1987) and relies on a scoring system to determine percent intestinal cells infected at a given interval. The second method, fecal oocyst production over time, relies on counting numbers of oocysts in feces of infected, immunosuppressed animals over many days or weeks (Heine et al. 1984). The third method is less labour intensive than the other two and relies on quantifying oocysts in the intestine of suckling mice at a given instant in time, following homogenization of entire intestinal tracts (Blagburn et al. 1991, Tilley et al. 1991a,b). Although this latter method is easier and more cost effective than the other two techniques, it still suffers from the same variability among and between experiments. However, the above results help explain some of this variability and demonstrates the importance of matching litters both by age and weight. Therefore, we believe that many of the previous studies involving suckling mice infected with *C. parvum* should be viewed cautiously. Past experiments involving the effects of pharmaceuticals on parasite development may be misleading in studies that compared parasite levels between mice of unequal ages. In addition, compounds affecting weight gain introduce a second variable by indirectly affecting parasite development.

In order to minimize some of the variability in the suckling mouse assay, we propose the following suggestions. First, the suckling mice be age matched at the time of inoculation. Depending upon mouse strain, preliminary studies may be necessary to find the optimal age when neonates should be infected. Second, that numbers of mice in each litter be consistent. We have found that an ICR dam can easily support 12 offspring, and groups comprising ≥ 10 individuals are intuitively pleasing and provide good standard deviations. Groups with smaller numbers of mice generally receive more milk per animal and gain weight faster than when higher numbers of neonates are nursed. Third, that researchers may wish to provide dams a mixture of offspring from different females. This randomizes any potential genetic differences among litters, and ICR females accept offspring from other females readily. Fourth, that all suckling mice be weighed prior to the experiment and the mean litter weights adjusted to within 0.1 g. Fifth, that litters be rotated between different females at 24 h intervals so that any effects of parental care, such as amount of milk produced, is randomized between groups. We have found there to be less chance of rejection if litters, rather than females themselves, are rotated. Inbred strains of mice may not be amenable to this type of manipulation.

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