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Individual subject meta-analysis of parameters for *Cryptosporidium parvum* shedding and diarrhoea in animal experimental models

A. D. ADELL[1], W. A. MILLER[1,2]*, D. J. HARVEY[3], E. VANWORMER[2], S. WUERTZ[4,5] AND P. A. CONRAD[1,2]

[1] *Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, CA, USA*
[2] *One Health Institute, School of Veterinary Medicine, University of California, Davis, CA, USA*
[3] *Department of Public Health Sciences, School of Medicine, University of California, Davis, CA, USA*
[4] *Department of Civil & Environmental Engineering, College of Engineering, University of California, Davis, CA, USA*
[5] *Singapore Centre on Environmental Life Sciences Engineering (SCELSE), School of Biological Sciences, and School of Civil and Environmental Engineering, Nanyang Technological University, Singapore*

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SUMMARY

Cryptosporidium is a zoonotic protozoan parasite with public health importance worldwide. The objectives of this study were to (1) conduct a meta-analysis of published literature for oocyst shedding and diarrhoea outcomes, and (2) develop recommendations for standardization of experimental dose–response studies. Results showed that for the outcome of oocyst shedding in faeces, the covariates 'experimental species', 'immunosuppression', 'oocyst dose' and 'oocyst dose' × 'age' were all significant ($P \leqslant 0{\cdot}05$). This study suggests that exposing mice, piglets, or ruminants, and using immunosuppressed experimental hosts, is more likely to result in oocyst shedding. For the outcome of diarrhoea in experimentally infected animal species, the key covariates 'experimental species', 'age' and 'immunosuppression' were significant ($P \leqslant 0{\cdot}2$). Therefore, based on the results of this meta-analysis, these variables should be carefully reported and considered when designing experimental dose–response studies. Additionally, detection of possible publication bias highlights the need to publish additional studies that convey statistically non-significant as well as significant results in the future.

Key words: *Cryptosporidium*, diarrhoea, experimental infection, individual subject meta-analysis, oocyst shedding.

INTRODUCTION

Cryptosporidium parvum is a zoonotic waterborne protozoan pathogen known to enter the environment through human and animal faeces [1]. Infection and gastrointestinal disease in humans can be caused by ingestion of small doses of *Cryptosporidium* spp. oocysts (10–30 oocysts) [2] directly from faeces or indirectly from contaminated water and food products [1]. Typically the diarrhoeal disease is self-limiting in immunocompetent individuals; however, this protozoa causes severe disease that can become chronic and lead to death in high-risk groups including immunocompromised (e.g. AIDS patients), elderly, and infant populations [3, 4]. This situation is even more

* Author for correspondence: Dr W. A. Miller, Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, One Shields Avenue, University of California, Davis, CA 95616, USA.
(Email: wamiller@ucdavis.edu)

critical for high-risk populations because currently there is limited effectiveness of anti-parasitic drugs to treat cryptosporidiosis in humans [1, 5, 6].

Animal and human dose–response models as well as *in vitro* cell culture have been widely used to evaluate C*ryptosporidium* spp. infectivity. At present, human infectivity studies are not considered to be practical because of the ethical concerns involved, while experimental animal models have been frequently used to estimate human infectivity and to provide information used for human health risk assessments. *In vitro* cell culture has been proposed as a potential alternative to animal assays because cultivation has the advantage of being cheaper, less time-consuming and does not raise the ethical concerns associated with using experimental animal models [7]. However, experimental animal model studies are often preferred when performing risk assessment, as they provide the most realistic scenario of what is happening with the pathogen once it enters the host. For this reason, this meta-analysis will focus on the diverse range of experimental animal model studies available in the peer-reviewed literature.

Numerous papers about animal model dose–response and infectivity experiments using diverse protocols have been published. Each of these experimental studies used different parameters, such as animal species, age, immune status, oocyst dose and detection methods to quantify oocysts in faeces and evaluate the infectivity of *Cryptosporidium* spp. as well as outcomes ranging from laboratory detection of oocyst shedding to clinical symptoms. Using diverse variables and protocols can be problematical in experimental studies because the variations in protocols between studies can affect the outcome of the experiments and thus their comparability [1]. Hence, a standardization of the experimental dose–response studies is required.

The present study aimed to make recommendations for the standardization of animal dose–response experiments by conducting a meta-analysis of individual subjects and exploring study design characteristics that cause heterogeneity between included studies. The hypothesis tested in this meta-analysis was that selected experimental factors of interest are associated with increased *C. parvum* and *C. hominis* oocyst shedding and diarrhoea. The results of this meta-analysis will be used to make recommendations for more standardized and comparable studies of the infectivity for these pathogenic parasite species, so that in future there may be fewer animal species and resources used to provide dose–response information more quickly and efficiently.

METHOD

Search strategy and selection of studies

The search of published experimental animal and/or human dose–response studies was performed using the electronic databases PubMed and Web of Science over the course of 160 hours from August 2009 to November 2009, and was updated during 1 week in August 2010 and March 2012. The process is described in Figure 1, with search criteria consisting of the following algorithms: (i) Protozoan infectious doses for humans, (ii) *Cryptosporidium* and dose response, (iii) *Cryptosporidium* and infectivity, (iv) *Cryptosporidium* and metaanalysis, (v) *Cryptosporidium* and meta-analysis, (vi) Cryptosporidiosis and experimental and model, and (vii) experimental and infection and *Cryptosporidium*. Unpublished studies (grey literature) were not included in this meta-analysis.

The articles identified by means of these search criteria were subject to further selection consisting of removal of the complete study or individuals within the articles that met any of the exclusion criteria (Table 1). One reviewer examined the titles and abstracts of all the articles found using the search criteria mentioned above. The full article for each of the relevant studies was then assessed by the same reviewer and a second reviewer was consulted when necessary. Only those articles that met at least one of the search criteria and none of the exclusion criteria were included in the data analysis. Furthermore, the reference lists of all included articles were searched for further possible papers but no additions were identified. Ethical approval was not required for this meta-analysis.

Data extraction

Data regarding the following variables for each individual animal were extracted and recorded from each article: (i) *C. parvum* genotype (*genotype*), (ii) isolate identification, such as Iowa for *C. parvum* (*isolate*), (iii) animal source of isolate (*isolate source*), (iv) whether oocysts were subject to animal passage before challenge (*animal passage*), (v) storage time of oocysts (in weeks) prior to inoculation of experimental host (*storage time*), (vi) method used to confirm

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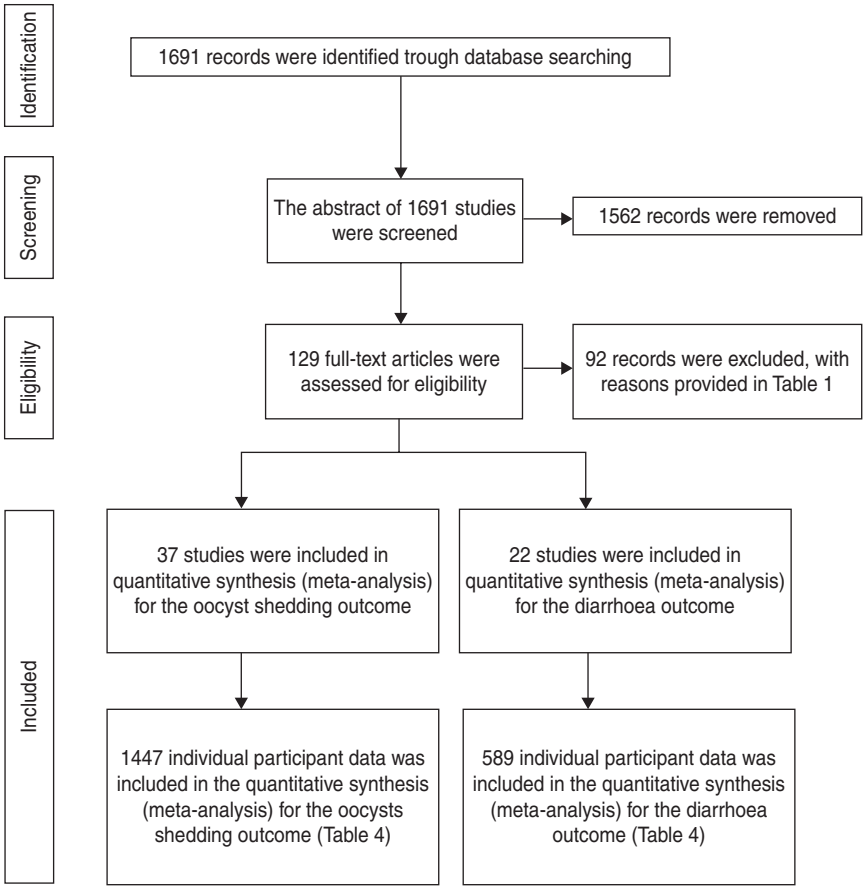

Fig. 1. Flow of information through the different phases of a systematic review.

oocyst viability prior to inoculation (*viability method*), (vii) animal species used as experimental host (*experimental species*), (viii) number of subjects per group inoculated, (ix) age of the experimental host (*age*), (x) whether the experimental host was subject to immunosuppression (*immunosuppression*), (xi) method of immunosuppression of the experimental host (*immunosuppression cause*), (xii) oocyst dose administered to the experimental host (*oocyst dose*), (xiii) administration route used for inoculation of experimental host with oocyst dose (*administration route*), (xiv) oocyst detection method in faeces of experimental hosts after inoculation (*detection method*) and number of animals that presented oocyst shedding and/or diarrhoea after inoculation.

The unit of analysis was individual animals, thus data for each variable were obtained for each animal and included in the analysis. If individual data for a variable was not reported, the variable was left blank. Table 2 provides information regarding the number of subjects and missing values by variable and study. The primary outcomes of interest were the presence of oocyst shedding and diarrhoea. An animal was considered to be shedding or have diarrhoea when the condition of the animal was indicated with the words diarrhoea or oocyst shedding in the study from which the information was extracted. For diarrhoea and oocyst shedding the classification of 'yes/no' was used. Initially, attempts were made to contact authors for clarification but due to lack of responses this approach was not systematically implemented throughout the entire study.

Classification of categorical variables and management of continuous variables

The extracted categorical variables were classified according to the information provided by the selected studies for each outcome (Table 3). As articles published before *C. hominis* was recognized as a separate species were included in this meta-analysis, the classifications 'genotype 1' and 'genotype 2', corresponding to the human genotype *C. hominis* and to the *C. parvum* zoonotic genotype, respectively, were used for the variable 'genotype' for both 'shedding' and 'diarrhoea' outcomes [1, 3, 8]. In the case of the

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Table 1. *Exclusion criteria for studies and number of articles excluded from meta-analysis*

Exclusion criteria	Articles excluded
1. Study was not an experimental dose–response experiment	32
2. *Cryptosporidium* spp. different than *C. parvum* or *C. hominis* were used	24
3. Infection was assessed only by histology on tissue sections	9
4. Experimental dose–response was measured in humans only	8
5. Oocyst dose was not provided or only a range was provided	7
6. Animal study groups that were subject to treatments other than the single inoculation/single outcome design used in this meta-analysis	5
7. Study was published in language other than English or Spanish	4
8. Number of animals used was not provided	2
9. Article was not available in electronic databases and in any of the University of California libraries	1

variable 'isolate source', the classification 'ruminant' was composed of cattle, deer, sheep and goats, whereas 'rodents' consisted of Siberian chipmunks and voles. For the variable 'viability method', the category 'excystation method' included the terms excystation, excystation rate and excystation percentage, and any staining methods used to determine the number of intact oocysts, including propidium iodine exclusion and 4k,6-diamidino-2-phenylindole (DAPI). The category '*in vivo*' consisted of studies using experimental infection of animals to confirm oocyst viability. Regarding the variable 'experimental species' the category 'other animals' included dogs, cats, birds and rabbits, the category 'ruminants' included lambs, goat kids and calves, and the class 'other rodents' was comprised of rats, gerbils, common voles (*Microtus arvalis*) and guinea pigs. The variable 'age' was categorized following published criteria (Table 4), which were consistent with the criteria used in the studies included in this meta-analysis. The classification of age was dependent on the experimental species because age can correspond to a different stage of maturity for each animal species. For instance, 2-month-old puppies are at a different stage of maturity than 2-month-old mice, and thus might have different susceptibilities to protozoan infection. The classification 'young' in the 'age' category consisted of newborn and weanling animals, while the classification 'adult' included only adult animals. For the variable 'immunosuppression cause', the class 'genetically' included, among others, animals whose immune system was weakened or abolished through genetic modification such as severe combined immune deficiency and nude mice; 'chemically' included all animals treated with drugs that cause a reduction in immune activity such as dexamethasone; and 'none' indicated that the experimental species was not subject to any immunosuppression method, and thus included immunocompetent animals. The variable 'administration route' was composed of the classifications 'gastric intubation' and 'oral inoculation'. 'Gastric intubation' included the terms gastric tube, intra-gastric route and stomach tube, while 'oral inoculation' included the terms orally, orogastric route and per os gavage. The variable 'detection method' was only used for the oocyst shedding outcome and it included four classes (Haemocytometer, Fluorescent microscopy, Flow cytometry and Light microscopy). 'Fluorescent microscopy' included direct fluorescent antibody (DFA), indirect fluorescent antibody (IFA) and fluorescent microscopy, whereas 'Light microscopy' was comprised of microscopic examination (by light or phase-contrast microscopy) of parasites (with or without concentration by flotation) that were stained by a variety of methods including Ziehl–Nielsen, nigrosine, acid fast, Heine, aniline-carbon-methyl violet, or any modification of these staining methods. The variables 'oocyst dose' and 'storage time' were continuous variables. As the values in the 'oocyst dose' variable ranged from 1 to 10^6 oocysts, the data for this variable were log transformed to make the distribution of the data more normal. The mean of the whole dose series (mean log scale = 4·5729) was subtracted from every dose value to centre the data. This enabled interpretation of the main effect in the presence of an interaction term at a

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Table 2. *Studies included for* Cryptosporidium parvum *and* C. hominis *meta-analysis of diarrhoea and shedding outcomes* (*chronological order*)

			Sample size (number of animals)										
Ref.	Year	Outcome	G[1]	IS[2]	AP[3]	ST[4]	VM[5]	ES[6]	A[7]	IS[8]	ISC[9]	AR[10]	DM[11]
[9]	1986	S[12]	190	190	190	190	NR[18]	190	190	190	190	190	190
[10]	1993	S[12]	NR[18]	NR[18]	NR[18]	NR[18]	20	20	20	20	20	20	20
[11]	1996	S[12]	NR[18]	50	50	50	NR[18]	50	50	50	50	50	50
[12]	1996	S[12]	NR[18]	9	9	9	NR[18]	9	9	9	9	9	9
[13]	1997	S[12]	NR[18]	NR[18]	5	5	5	5	5	5	5	5	5
[14]	1997	S[12]	NR[18]	NR[18]	1	NR[18]	NR[18]	1	1	1	1	1	1
[15]	1998	S[12]	NR[18]	18	18	18	NR[18]	18	18	18	18	18	18
[16]	2000	S[12]	14	14	14	NR[18]	NR[18]	14	14	14	14	NR[18]	14
[17]	2001	S[12]	NR[18]	4	4	NR[18]	NR[18]	4	4	4	4	4	4
[8]	2002	S[12]	NR[18]	102	102	102	102	102	102	102	102	102	102
[18]	2003	S[12]	NR[18]	130	130	130	NR[18]	130	130	130	130	130	130
[19]	2003	S[12]	14	14	14	14	14	14	14	14	14	14	14
[20]	2003	S[12]	8	8	NR[18]	NR[18]	NR[18]	8	8	8	8	8	8
[21]	2005	S[12]	22	22	NR[18]	22	NR[18]	22	22	22	22	22	22
[22]	2005	S[12]	14	32	32	NR[18]	NR[18]	32	32	32	32	32	32
[23]	2007	S[12]	NR[18]	180	180	180	180	180	180	180	180	180	180
[24]	1992	D[13]	NR[18]	48	48	48	48	48	48	48	48	48	NR[18]
[25]	1990	S and D[14]	NR[18]	17[17]	17[17]	NR[18]	NR[18]	17[17]	17[17]	17[17]	17[17]	17[17]	17[15], NA[19]
[26]	1992	S and D[14]	NR[18]	NR[18]	140[15], 40[16]	140[15], 40[16]	NR[18]	140[15], 40[16]	140[15], 40[16]	140[15], 40[16]	140[15], 40[16]	140[15], 40[16]	140[15], NA[19]
[27]	1993	S and D[14]	NR[18]	40[17]	40[17]	NR[18]	NR[18]	40[17]	40[17]	40[17]	40[17]	40[17]	40[15], NA[19]
[28]	1993	S and D[14]	NR[18]	5[17]	5[17]	5[17]	NR[18]	5[17]	5[17]	5[17]	5[17]	5[17]	5[15], NA[19]
[29]	1994	S and D[14]	7[15], NR[18]	25[15],18[16]	25[15],18[16]	NR[18]	NR[18]	25[15],18[16]	25[15],18[16]	25[15],18[16]	25[15],18[16]	NR[18]	25[15], NA[19]
[30]	1997	S and D[14]	NR[18]	12[17]	12[17]	12[17]	12[17]	12[17]	12[17]	12[17]	12[17]	12[17]	12[15], NA[19]
[31]	1997	S and D[14]	NR[18]	11[17]	11[17]	NR[18]	NR[18]	11[17]	11[17]	11[17]	11[17]	11[17]	11[15], NA[19]
[32]	1997	S and D[14]	NR[18]	NR[18]	26[17]	NR[18]	NR[18]	26[17]	26[17]	26[17]	26[17]	26[17]	NR[18], NA[19]
[33]	1997	S and D[14]	NR[18]	8[17]	8[17]	8[17]	8[17]	8[17]	8[17]	8[17]	8[17]	8[17]	8[15], NA[19]
[34]	1998	S and D[14]	NR[18]	80[17]	80[17]	NR[18]	NR[18]	80[17]	80[17]	80[17]	80[17]	80[17]	NR[18], NA[19]
[35]	1998	S and D[14]	NR[18]	NR[18]	70[17]	NR[18]	NR[18]	70[17]	70[17]	70[17]	70[17]	28[17]	70[15], NA[19]
[36]	1998	S and D[14]	NR[18]	26[17]	NR[18]	NR[18]	NR[18]	26[17]	26[17]	26[17]	26[17]	26[17]	26[15], NA[19]
[37]	1999	S and D[14]	NR[18]	13[17]	13[17]	NR[18]	NR[18]	13[17]	13[17]	13[17]	13[17]	13[17]	13[15], NA[19]
[38]	2002	S and D[14]	NR[18]	2[17]	2[17]	2[17]	NR[18]	2[17]	2[17]	2[17]	2[17]	2[17]	2[15], NA[19]
[39]	2002	S and D[14]	35[17]	35[17]	35[17]	35[17]	35[17]	35[17]	35[17]	35[17]	35[17]	35[17]	35[15], NA[19]
[40]	2003	S and D[14]	14[17]	14[17]	14[17]	14[17]	NR[18]	14[17]	14[17]	14[17]	14[17]	14[17]	14[15], NA[19]
[41]	2003	S and D[14]	11[17]	11[17]	11[17]	11[17]	NR[18]	11[17]	11[17]	11[17]	11[17]	11[17]	11[15], NA[19]
[42]	2003	S and D[14]	8[17]	8[17]	8[17]	NR[18]	NR[18]	8[17]	8[17]	8[17]	8[17]	8[17]	8[15], NA[19]
[43]	2005	S and D[14]	82[17]	42[17]	82[17]	82[17]	82[17]	82[17]	82[17]	82[17]	82[17]	82[17]	82[15], NA[19]
[44]	2009	S and D[14]	20[17]	20[17]	20[17]	NR[18]	20[17]	20[17]	20[17]	20[17]	20[17]	20[17]	20[15], NA[19]
[45]	2009	S and D[14]	3[17]	3[17]	3[17]	3[17]	NR[18]	3[17]	3[17]	3[17]	3[17]	3[15], NR[18]	3[15], NA[19]

[1] Genotype, [2] isolate source, [3] animal passage, [4] storage time (weeks), [5] viability method, [6] experimental species, [7] age, [8] immunosuppression, [9] immunosupression cause, [10] administration route, [11] experimental method, [12] shedding outcome, [13] diarrhoea outcome, [14] shedding and diarrhoea outcome, [15] number of individuals for shedding outcome, [16] number of individuals for diarrhoea outcome, [17] number of individuals for both outcomes, [18] information was not reported in study, thus no individual data was included, [19] does not apply.

Table 3. *Classification of categorical variables for oocyst shedding and diarrhoea outcomes*

	Shedding outcome			Diarrhoea outcome		
Variable	Classification of variables	No. of studies	No. of individuals	Classification of variables	No. of Studies	No. of individuals
Genotype	Genotype 1	5	331	Genotype 1	3	57
	Genotype 2	12	199	Genotype 2	6	116
	Porcine	1	14			
Isolate source	Ruminant	19	450	Ruminant	14	315
	Rodent	2	32	Human	6	98
	Human	11	367			
	Piglet	2	194			
Animal passage	No	16	419	No	9	153
	Yes	22	952	Yes	14	410
Viability method	Excystation rate	7	453	Excystation rate	3	137
	In vivo	3	25	*In vivo*	3	68
Experimental species	Other animals	7	101	Other animals	1	26
	Other rodents	6	196	Other rodents	2	102
	Mice	15	940	Mice	6	276
	Piglet	5	52	Piglet	5	81
	Ruminants	15	158	Ruminants	11	104
Age	Young	26	883	Young	18	240
	Adult	15	564	Adult	8	349
Immunosuppression	No	30	796	No	19	328
	Yes	12	651	Yes	7	261
Immunosuppression cause	Chemically	5	422	Chemically	3	112
	Genetically	8	229	Genetically	4	149
	None	30	796	None	19	328
Administration route	Gastric intubation	9	417	Gastric intubation	6	204
	Oral inoculation	27	946	Oral inoculation	14	274
Detection method	Haemocytometer	8	282	Not analysed		
	Fluorescent microscopy	5	270			
	Flow cytometry	1	18			
	Light microscopy	21	771			

meaningful value (the mean) [63]. For instance, in the multivariable model the main effect of 'age' on the interaction 'age' × 'oocyst dose' corresponds to a difference between young and adult at a mean dose. If the mean of the whole dose series was not subtracted, this comparison would have been the difference between young and adult at a log dose of zero (dose of 1). Log transformation of the 'storage time' (weeks) variable did not improve the model fit.

Statistical analyses

In this meta-analysis a one-step approach was used to analyse where the individual subject data from all studies were modeled while accounting for the clustering of participants within studies [64]. The statistical analyses involved construction of histograms, bivariate analysis and multivariable analysis. The histograms were used to determine the distribution of each variable of interest. Each variable was then evaluated using Mantel–Haenzel and Generalized Linear Mixed Models (GLIMMIX) bivariate analyses for categorical and continuous variables, respectively, to determine the association between each one of them alone and each outcome. Mantel–Haenzel and GLIMMIX were chosen because they take into account the correlation between matching variables and the heterogeneity of effects among subjects, respectively [63]. In other words, both analyses take into consideration the correlation between individuals that were included in the same study. $P \leqslant 0{\cdot}2$ was considered significant for the bivariate analyses as they do not take into account the effect of covariates, thus a variable might have a statistically non-significant

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Table 4. *Criteria used to determine age categories for experimental species* (*listed alphabetically*)

Animal species	Criteria used
Cattle	Newborn: <21 weeks Weanling: 21–64 weeks [46] Adult: ⩾65 weeks [47]
Cats	Newborn: <8 weeks Weanling: 8–25 weeks [48] Adult: ⩾26 weeks [47]
Chickens	Newborn: 0–1 weeks [49]
Common voles	Adult: ⩾4 weeks [50]
Dogs	Newborn: <8 weeks Weanling: 8–29 weeks [51] Adult: ⩾30 weeks [47]
Geese	Young <104 weeks Adult ⩾104 weeks [52]
Gerbils	Newborn: <4 weeks Weanling: 4–7 weeks [53] Adult: ⩾8 weeks [54]
Goats	Newborn: <13 weeks [55] Weanling: 13–47 weeks [56] Adult: ⩾48 weeks [55]
Guinea pigs	Newborn: <3 weeks Weanling: 3 weeks [57]
Mice	Newborn: <3 weeks Weanling: 3 weeks [58] Adult: ⩾4 weeks [47]
Pigs	Newborn: <3 weeks Weanling: 4–21 weeks [59] Adult: ⩾22 weeks [47]
Rabbits	Newborn: <4 weeks Weanling: 4–17 weeks [60] Adult: ⩾18 weeks [47]
Rats	Newborn: 3 weeks Weanling: 3–7 weeks [61] Adult: ⩾8 weeks [47]
Sheep	Newborn: <8 weeks Weanling: 8–21 weeks [62] Adults: ⩾22 weeks [47]

Weanling was merged with newborn to create the classification 'young' in the 'age' variable.

result due to confounder effect and not for not presenting an association with the outcome. For multivariable analysis $P \leqslant 0{\cdot}05$ was considered significant.

Significant bivariate predictors ($P \leqslant 0{\cdot}2$) were included in the GLIMMIX multivariable analysis to determine the association between these variables jointly and the outcome of interest. For this analysis a binomial distribution with a logit function and an exchangeable covariance were assumed, and the study from which the data were extracted was assigned as the random effect [63]. The multivariable model was assembled using a forward step procedure to incorporate one significant variable of interest into the model at a time and the reference categories for this analysis were chosen for biologically significant comparisons. Once the initial model was complete, confounders were assessed. A confounder was identified as any variable that caused a >10% change in the coefficient estimate for at least one of the main effects when placed in the model [65]. The term 'confounder' rather than 'effect modifier' is used throughout the paper because the term 'confounder' is an epidemiological term specifically describing a variable associated with a change in the coefficient estimate of at least one variable when placed in the model, while the term 'effect modifier' often describes a change of estimate as an interaction between two variables [63]. Interaction terms were then incorporated in the model, and statistically significant interactions were retained in the final models. Scatter plots and box plots were created to assess the correlation between the variables that were included in the final models. In the case where there was correlation between variables in the model, the variable that provided the smaller confidence interval range was retained in the model. The selection of the final models was based on the presence of all four key variables corresponding to 'experimental species', 'age', 'oocyst dose' and 'immunosuppression' or 'immunosuppression cause', and having a combination of variables that generated narrower confidence intervals or greater precision. The fit of the model was assessed by the ratio of the generalized χ^2 statistic and its degrees of freedom (generalized χ^2/D.F.), which could not exceed a value of 1, and residual plots. The statistical software JMP 9 (SAS Institute Inc., USA) and SAS 9.3 (SAS Institute Inc.) were used to perform all the analyses.

Publication bias was assessed using funnel plots, Egger's test of the intercept, and Duval & Tweedie's trim-and-fill procedure. The funnel plot provides a visual sense of the relationship between effect size and study size (standard error or precision) [66]. In the absence of publication bias, the studies will be distributed symmetrically about the combined effect size, otherwise the plot would show a higher concentration of studies on one side of the mean than on the other [66]. Furthermore, as significant heterogeneity (variation among study outcomes) can lead to spurious publication bias results, Cochran's Q test and I^2 statistics were used to assess the heterogeneity in the studies included in this meta-analysis. A significance

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Table 5. *Bivariate analysis results: shedding outcome after Cryptosporidium infection*

Variable	*P* value	Statistical association between variable and oocyst shedding (yes/no)*
Genotype†	<0·0001	Yes
Isolate source†	0·0207	Yes
Animal passage†	0·0001	Yes
Storage time (weeks)‡	<0·0001	Yes
Viability method†	Not calculated§	
Experimental species†	<0·0001	Yes
Age†	0·4585	No
Immunosuppression†	0·0179	Yes
Immunosuppression cause†	<0·0001	Yes
Oocyst dose (log scale)‡	<0·0001	Yes
Administration route†	0·0894	Yes
Detection method†	Not calculated§	

* Significance level ⩽0·2.
† Mantel–Haenzel bivariate analyses taking into account the correlation between studies.
‡ GLIMMIX bivariate analyses.
§ Analysis did not compute estimates due to paucity of data.

level of ⩽0·05 was used to assess publication bias, while $P \leqslant 0{\cdot}1$ was used to assess heterogeneity. Comprehensive Meta-Analysis software version 2 (Biostat^{TM}, USA) was used to assess both publication bias and heterogeneity in the studies.

RESULTS

Search strategy and selection of studies

The initial search identified 1691 potentially relevant studies. Subsequent to reviewing the abstracts, 129 studies were considered for further screening, from which 92 were not eligible since at least one of the exclusion criteria (Table 1) was met. After examination of the full text, 37 studies were included for the oocyst shedding outcome while 22 were incorporated for the diarrhoea outcome. The studies included in this meta-analysis were published between 1986 and 2009 and are shown in Table 2. The total number of individuals included in this meta-analysis was 1447 individuals for the shedding outcome and 589 individuals for the diarrhoea outcome. The flow chart for the selection of articles is shown in Figure 1.

Analysis for oocyst shedding outcome

The bivariate analysis showed that the covariates 'genotype', 'isolate source', 'animal passage', 'storage time', 'experimental species', 'immunosuppression', 'immunosuppression cause', 'oocyst dose' and 'administration route' were modestly associated with the outcome of oocyst shedding in faeces ($P \leqslant 0{\cdot}2$) (Table 5). These variables were then incorporated in the multivariable analysis which provided one multivariable model that best fulfilled the selection criteria mentioned in the Methods section. The multivariable model (Table 6) showed that the variables 'animal passage', 'experimental species', 'immunosuppression' and 'oocyst dose', and the 'oocyst dose'×'age' interaction have at least one level that is significant (significance level ⩽0·05). Conversely, the variables 'age' and 'administration route' were not significant; however, they were incorporated in the model because both were identified as potential confounders as they caused a >10% change in the estimate of the variable 'experimental species'.

The multivariable model indicated that experimental hosts inoculated with oocysts subject to animal passage had 5·54 times higher odds of oocyst shedding than experimental hosts infected with oocysts that were not subject to passage [$P < 0{\cdot}001$, 95% confidence interval (CI) 2·59–11·89]. In the case of the variable 'experimental species', 'other animals' was the only category that was statistically significantly different from 'mice', indicating that 'other animals' as experimental hosts were <0·001 times less likely to

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Table 6. *Multivariable model for oocyst shedding outcome after* Cryptosporidium *infection**

Variable	Reference	Categories	Odds ratio estimate	95% Wald confidence interval	*P* value
Animal passage	No	Yes	5·54	(2·59–11·89)	<0·0001
Experimental species	Mice	Other animals	<0·001	(<0·001–0·01)	<0·0001
		Other rodents	0·62	(0·03–15·06)	0·77
		Piglets	1·83	(0·02–177·87)	0·80
		Ruminants	0·06	(0·001–3·51)	0·18
Age	Young	Adult	2·35†	(0·09–59·89)	0·60
Immunosuppression	Yes	No	0·38	(0·16–0·89)	0·03
Oocyst dose	Continuous variable		9·06‡	(5·29–15·51)	<0·0001
Administration route	Orally	Gastric intubation	0·20	(0·005–7·76)	0·38
Oocyst dose × age		Adult	2·37§	(1·08–5·19)	0·004

* Pseudo-Akaike's Information Criteria = 7638·25. Generalized χ^2 statistic and its degrees of freedom (gen. χ^2/D.F.) = 0·59.
† The odds ratio estimate corresponds to adult *vs.* young at the mean oocyst dose.
‡ The odds ratio estimate corresponds to an increase in oocyst dose in young animals.
§ The odds ratio estimate corresponds to an increase in oocyst dose in adult animals.

shed oocysts than 'mice' ($P<0·001$, 95% CI <0·001–0·01). Figure 2*a* shows the range of doses compared to the odds of detecting shedding; thus showing how the choice of the experimental animal species used impacts the dose being investigated. The plot indicated that there is an increase in odds of oocyst shedding at higher doses for each experimental species, with 'piglets' having the greatest increase in odds while 'ruminants' had the lowest odds. Experimental hosts that were not immunosuppressed were 0·38 times less likely to have oocyst shedding than immunosuppressed animals, regardless of the cause of immunosuppression ($P=0·03$, 95% CI 0·16–0·89). For each unit of change in the log of oocyst dose administered to young experimental hosts, the odds of having oocyst shedding increased 9·06 times ($P<0·0001$, 95% CI 5·29–15·51). In adult experimental hosts, a change of one unit in the log of oocyst dose administered increased the odds by 2·37 times ($P=0·004$, 95% CI 1·08–5·19). Figure 2*b* shows the ranges of doses compared to the odds of detecting shedding and how choosing young experimental animals had a higher impact on the dose being investigated compared to adult experimental animal.

The fit of the models was assessed by the ratio value for generalized χ^2/D.F. and residual plots. The generalized χ^2/D.F. for the multivariable model was 0·59, indicating that the model had good fit. The residual plots (not shown), in which the residual value was plotted against the linear predictor, showed that two observations in the model had large residuals ($\geqslant 15$) and were possible outliers from the cluster of observations. Removing the individuals that had large residuals did not change the significance of the other variables of the model, thus they were kept in the model.

In this meta-analysis we found evidence of possible publication bias for the oocyst shedding outcome. The funnel plot had a higher concentration of studies to the right of the mean (Fig. 3). Egger's regression test suggested a significant association between study size and study effect with a *P* value of 0·003 (significance level $\leqslant 0·05$). Duval & Tweedie's trim-and-fill method suggested adding 12 studies to the left side of the funnel plot, which under the random-effects model would shift the point estimate of the prevalence of oocyst shedding of all the studies included in this meta-analysis from 0·84 (95% CI 0·76–0·89) to 0·72 (95% CI 0·62–0·80), improving the estimate. However, the results of Cochran's *Q* test indicated significant heterogeneous results ($P<0·001$) in different studies and the I^2 statistic determined that 84% of variation across studies was due to significant heterogeneity rather than random chance.

Analysis for diarrhoea outcome

As the data for diarrhoea outcome were too sparse, the same analysis performed for the shedding outcome could not be performed for the diarrhoea outcome. For instance, the bivariate analysis was performed considering the data as a pool across studies to estimate associations rather than accounting for the correlation between studies as done for the shedding outcome. The bivariate analysis showed that for the outcome of diarrhoea in exposed animals, the

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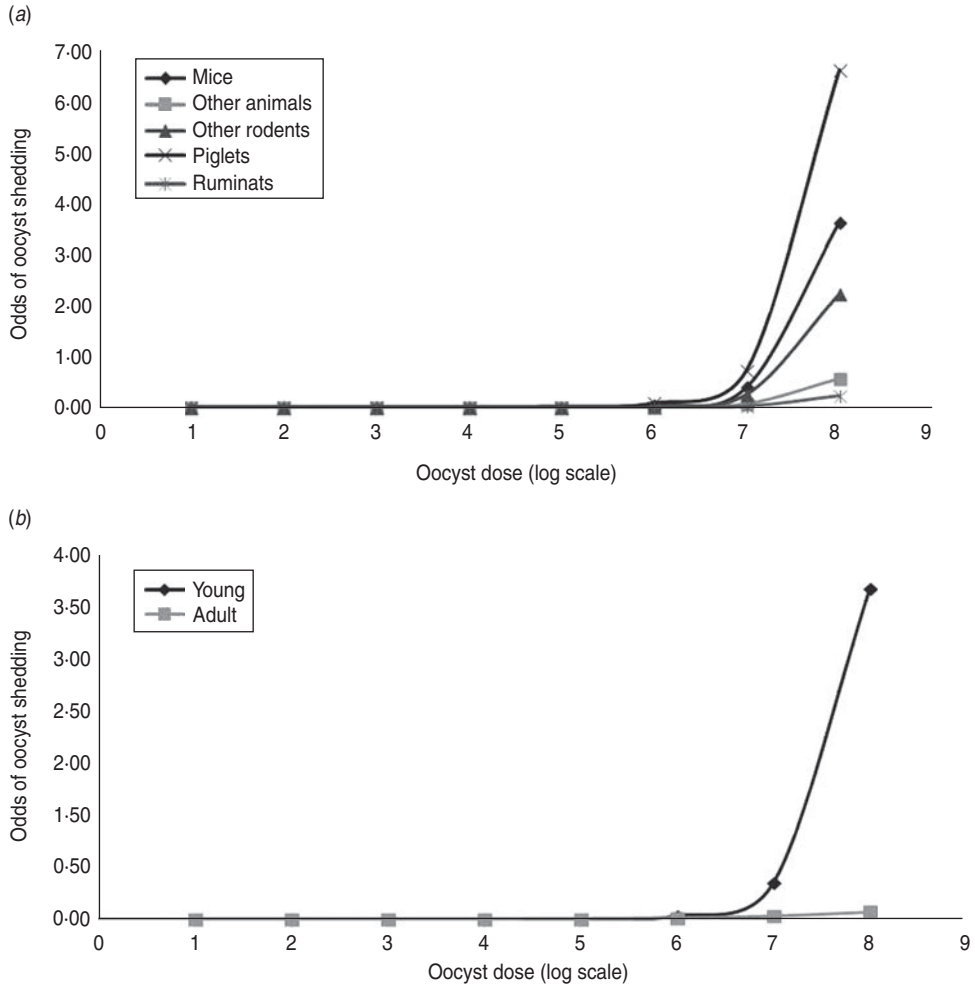

Fig. 2. (*a*) Range of oocyst dose (log scale) by odds of oocyst shedding by 'Experimental Species'. (*b*) Range of oocyst dose (log scale) by odds of oocyst shedding by age category.

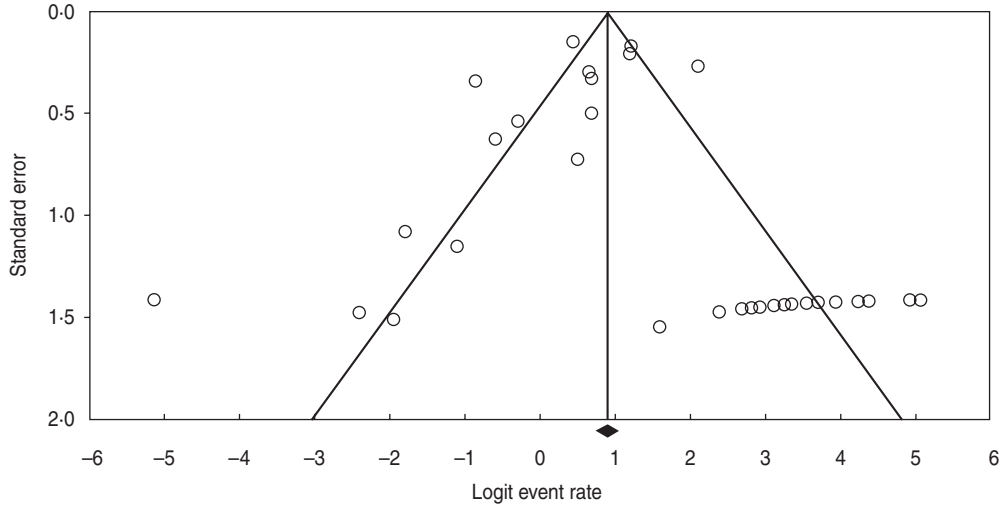

Fig. 3. Funnel plot of standard error by logit event rate for oocyst shedding outcome after *Cryptosporidium* infection showing possible publication bias.

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Table 7. *Bivariate analysis results: diarrhoea outcome after Cryptosporidium infection*

Variable	*P* value	Statistical association between variable and presence of diarrhoea (yes/no)*
Genotype†	0·0004	Yes
Isolate source†	0·005	Yes
Animal passage†	<0·0001	Yes
Storage time (weeks)‡	0·84	No
Viability method†	<0·0001	Yes
Experimental species†	<0·0001	Yes
Age†	<0·0001	Yes
Immunosuppression†	<0·0001	Yes
Immunosuppression cause†	<0·0001	Yes
Oocyst dose (log scale)‡	Analysis did not converge	
Administration route†	<0·0001	Yes
Detection method†	Not analysed	

* Significance level ⩽0·2
† Mantel–Haenzel bivariate analyses not taking into account the correlation between studies.
‡ GLIMMIX bivariate analyses.

covariates 'genotype', 'isolate source', 'animal passage', 'viability method', 'experimental species', 'age', 'immunosuppression', 'immunosuppression cause', and 'administration route' were significant ($P \leqslant 0{\cdot}2$) (Table 7). Due to the smaller number of studies reporting on the outcome of diarrhoea and variability in the reporting of these potential covariates across studies, it was not possible to create a multivariable model for this outcome. In this meta-analysis we found evidence of absence of publication bias for the diarrhoea outcome. The funnel plot was noticeably symmetric with a homogeneous concentration of studies on both sides of the mean (Fig. 4). Egger's regression test suggested a non-significant association between study size and study effect with a *P* value of 0·12 (significance level ⩽0·05). Duval & Tweedie's trim-and-fill method did not add any additional studies to the funnel plot, therefore maintaining the point estimate of the prevalence of diarrhoea of all the studies included in this meta-analysis (point estimate 0·44, 95% CI 0·19–0·73). The results of Cochran's *Q* test indicated significant heterogeneous results ($P < 0{\cdot}001$) among different studies and the I^2 statistic determined that 85% of variation across studies was due to significant heterogeneity rather than random chance.

DISCUSSION

This is the first meta-analysis to evaluate the effects of multiple experimental covariates on oocyst shedding or diarrhoea as indicators of *C. parvum* and *C. hominis* infection. The results obtained in this meta-analysis identified covariates that potentially cause heterogeneity between the results of the dose results experiments and suggest that the type of experimental animal host, age class of the experimental host, host immunosuppression status, and oocyst dose administered can all significantly impact the incidence of oocyst shedding and diarrhoea. Based on the results of this meta-analysis, it would be appropriate to make the following recommendations for future dose–response experiments on *C. parvum* and *C. hominis* when assessing infection by means of the presence of oocyst shedding in experimentally infected animals: (i) consider using 'mice', 'other rodents', 'ruminants' and 'piglets' as experimental host rather than dogs, cats, rabbits and birds, (ii) consider using immunosuppressed experimental animals rather than inmunocompetent animals, (iii) consider 'age' and 'oocyst dose' together, and (iv) subject the oocysts to animal passage before inoculation into experimental host. These covariates should be considered when designing experimental dose–response studies, as once the design of the dose–response studies are more standardized, they will provide better information and more comparable results for more accurate risk assessments that consider infection as the outcome.

In the case of assessing infection by means of the presence of diarrhoea in experimentally infected animals, the following recommendations are suggested when designing an experimental study: consider and report (i) the genotype of the *Cryptosporidium* being inoculated into the experimental host, (ii) the original source of the oocysts being inoculated, (iii) if oocysts were subject to animal passage before inoculation of experimental host or not, (iii) animal species that will be used as a experimental host, (iv) the age of the experimental host, (v) the immune status of the experimental host, (vi) the method used to cause the immunosuppression, and (vii) the administration route used for inoculation of oocysts into the experimental host. Based on the results of this meta-analysis, more experimental studies in animal models should be conducted to assess infectivity of *Cryptosporidium* by means of diarrhoea and the covariates mentioned above should be considered when

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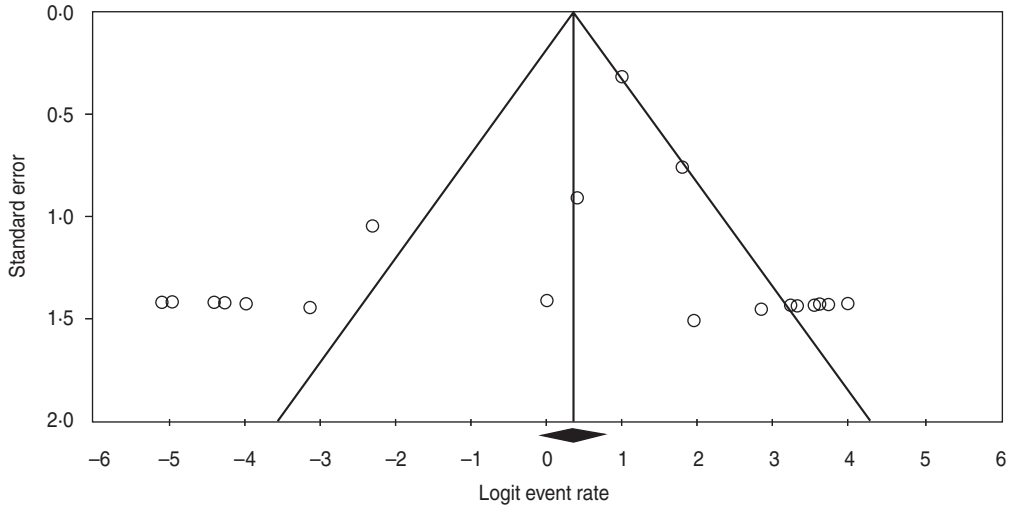

Fig. 4. Funnel plot of standard error by logit event rate for diarrhoea outcome after *Cryptosporidium* infection showing no publication bias.

designing these experimental dose–response studies. These studies will provide better information and more comparable results for risk assessments that consider illness as an outcome.

Oocyst shedding

The multivariable results presented above indicated that the key variables 'experimental species', 'immunosuppression' and 'oocyst dose' have at least one category with a statistically significant difference compared to the reference category. In the multivariable model, 'other animals' was the only category that had a statistically significant protective effect against oocyst shedding when compared to 'mice'. 'Other animals' was composed of dogs, cats, rabbits and birds; however, relatively few animals of these species were used in the studies included in this meta-analysis, providing a possible explanation for the low odds ratio estimates obtained in the multivariable analysis. The results of the multivariable analysis suggested that mice were more likely to shed oocysts after being experimentally infected compared to other animals; therefore, it would be advisable to use mice as the experimental host to increase the probability of oocyst shedding.

A non-statistically significant protective effect was also observed when comparing both 'other rodents' and 'ruminants' with 'mice' in the model. Although the estimate indicates a protective effect, there is not enough information from the published studies to suggest that these groups are at any less risk of oocyst shedding than 'mice'. However, regardless of 'other rodents' not being statistically different from 'mice', it is important to take into consideration that some species might vary in their susceptibilities to different *Cryptosporidium* spp.; for instance, piglets and immunosuppressed gerbils are the only species that have been reported to be susceptible to *C. hominis* [1, 8, 41, 42, 43], while a wide variety of animals, such as cattle, sheep, goats and humans, are susceptible to *C. parvum* [1, 3]. Thus, choosing to use mice, other rodents, or piglets as experimental hosts would be appropriate only for some study objectives, such as evaluating the infectivity of *C. hominis* or *C. parvum* by means of oocyst shedding. Nevertheless, further meta-analyses should be performed in the future when additional studies, using animal species other than mice (e.g. dogs, cats, rabbits, birds) as the experimental host, are published to obtain more accurate odds estimates.

Furthermore, it would have been interesting to evaluate the interaction term 'experimental species' × 'oocyst dose' to investigate how the dose and the choice of the experimental species impacts the odds of oocyst shedding. Unfortunately, the data were too sparse for the analysis to provide estimates for this interaction in this meta-analysis. Figure 2*a* was created to evaluate the range of doses compared to the odds of detecting shedding; thus showing how the choice of the experimental animal species used impacts the dose being investigated. As expected there is an increase in odds of oocyst shedding at higher doses for each experimental species, with 'piglets' having the greatest increase in odds while 'ruminants' had the lowest odds. These host-specific differences have

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implications for transmission routes and could lead to management recommendations such as prioritizing selection of 'lower risk' animal host for high-risk transmission settings such as petting zoos and classrooms. Based on these results, it is recommended that more experimental studies in animal models be conducted to evaluate the effect of the interaction term 'experimental species' × 'oocyst dose'.

In the multivariable model, the immunocompetent experimental hosts were less likely to shed oocysts than immunosuppressed animals, regardless of the immunosuppression cause. This result was expected as the human population at risk for severe disease is immunosuppressed individuals [3, 4]. Furthermore, it has been reported that immunosuppression has to be maintained, otherwise the immunosuppressed adult mice and rats will recover from their *C. parvum* infection, suggesting that immunosuppression increases and facilitates the development for cryptosporidiosis infection [1, 67]. The results provided by the multivariable model indicated that it is advisable to use immunosuppressed experimental hosts to ensure that study animals shed oocysts.

Several studies in which different doses of *Cryptosporidium* oocysts have been administered to animal models have been published. The results of these studies indicate that the percentage of animals shown to be infected based on the presence of *Cryptosporidium* in intestinal tissue sections or faecal samples increases when the number of oocysts administered is higher [21, 43, 68, 69]. The results obtained in the multivariable model were consistent with the findings of these published studies, indicating that when a higher dose of oocysts is administered, the odds of having oocyst shedding increases.

Pathogen shedding patterns for newborns, weanlings, and adults can be quite different across host species. For instance, it has been reported that mice and other animals generally develop a poorly characterized natural resistance to *C. parvum* infection before weaning [70], and so may be less susceptible as weanlings or adults. Unfortunately, in this meta-analysis there were not enough studies to analyse newborn and weanling separately, thus both categories had to be merged and analysed as 'young'. Based on the meta-analysis results, 'young' animals were not statistically different from 'adult' animals with regard to oocyst shedding when the mean of the log of oocysts was administered to the experimental host. In contrast, mice have been shown to be significantly more likely to be susceptible to infection, thus to oocyst shedding, when they are young, particularly aged <10 days [12]. Tarazona *et al*. [34] reported that mice infected at age 3 weeks had a shorter prepatent period and shed oocysts for a longer period of time compared to 4-week-old mice, but the number of subjects in the 3- and 4-week age groups that shed oocysts did not vary.

Of note, the significant 'age' × 'oocyst dose' interaction and Figure 2*b* suggest that an increase in the log of oocysts administered has a larger impact on oocyst shedding in 'young' experimental hosts compared to 'adult' experimental hosts, indicating a larger difference between the age groups as the administered dose increases. Thus, differences by 'age' depend on the 'oocyst dose', and it is advisable to consider these two variables together when designing experimental studies. For instance, only small volumes can be delivered accurately into the oesophagus of newborn or infants without inflicting injury [1], and thus a successful administration and evaluation on the infectivity of higher doses of oocysts might be more complicated for this age group. Adult animals, on the other hand, can receive both small and large volumes and oocyst doses, and therefore the probability of infection will vary with the dose administered. Moreover, the difference between age groups has public health importance as supported by numerous observational epidemiological studies showing that the young are the most affected [1]. Given that young individuals, in particular calves, represent a potential public health risk of shedding large numbers of oocysts through their faeces [1], management practices may best be targeted to minimize calf risk of oocyst exposure and to reduce calf contact with other animals and humans who may be susceptible.

The variables, such as 'genotype', 'isolate source', 'animal passage', 'storage time', 'viability method', and 'detection method' were identified as covariates for oocyst shedding in the bivariate analysis. However, information on these variables was not reported for all studies, which made it a challenge to consider them jointly in a multivariable model. Based on the results of this meta-analysis, it is highly recommended that experimental studies provide more reporting on these variables. Once more experimental studies providing information regarding these variables are published, it would be interesting to incorporate them in the same model as 'experimental species', 'immunosuppression', 'age' and 'oocyst dose' to ascertain the independent effect of these possible covariates on the presence of oocyst shedding.

In the multivariable model, oocysts that were subject to animal passage had higher odds of generating oocyst shedding in the experimental host than those oocysts that did not have any passage in animals. These results suggest that *Cryptosporidium* oocysts should be subject to passage in another animal before being inoculated into the experimental host to increase the likelihood of oocyst shedding. Animal passage of parasites is the standard practice used to maintain infectivity of oocysts as there are no optimal laboratory methods widely available [1].

In this meta-analysis, we found evidence of possible publication bias for the oocyst shedding outcome. This finding can be explained by the fact that smaller studies are more likely to be published if they have larger than average effects, which makes them more likely to meet the criterion for statistical significance [66]. The addition of studies by the trim-and-fill procedure improved the point estimate of the prevalence of oocyst shedding of all the studies included in this meta-analysis; however, the adjusted estimate is similar to the original effect and thus indicates that the reported trends may be valid. Caution is advised when interpreting these results because there is evidence of high heterogeneity in the studies, thus precluding a full evaluation of publication. These findings suggest that it is important to publish those studies that have negative or non-significant results as well those that have significant or positive results in order to reduce or avoid the bias.

Diarrhoea

Fewer articles were included in the analysis for the diarrhoea outcome compared to the oocyst shedding outcome because not many publications reported diarrhoea. For the diarrhoea outcome, all of the variables except 'oocyst dose' were modestly associated ($P<0{\cdot}2$) with the presence of diarrhoea in the bivariate analysis. The analysis for 'oocyst dose' did not converge due to small sample size and because diarrhoea was only present in animals given high oocyst doses, while the majority of animals without diarrhoea were given low doses. This finding could be explained by higher oocyst doses causing diarrhoea while low doses might be conferring short-term immunity and therefore protection. This is consistent with the result obtained by Moss *et al.* [71] which suggested that characteristic antibody responses develop following *C. parvum* infection and that persons with pre-existing antibodies may be less likely to develop illness. Unfortunately in the current study, there was not enough information on the dose variable for the bivariate analysis to provide statistical results regarding associations between oocyst dose administered to the experimental host and diarrhoea outcome. The low number of articles reporting diarrhoea is not surprising because the majority of infection studies have been in mice, an animal model not reported to have diarrhoea following oocyst exposure [72]. It should be noted that while the diarrhoea outcome is of clinical relevance, the presence of asymptomatic infected individuals is a limitation of using diarrhoea to represent infectious status.

In this meta-analysis we did not find evidence of publication bias for the diarrhoea outcome, in contrast to the shedding outcome. A lack of publication bias for the diarrhoea outcome could be because most articles that reported diarrhoea actually reported shedding as the primary outcome of interest, and so publication decision may not have been linked to significant findings with regard to diarrhoea. It should also be kept in mind that the high heterogeneity observed across studies for both outcomes limits our ability to truly evaluate publication bias. Based on the results, it is crucial that more experimental studies in animal models are conducted to assess infectivity of *Cryptosporidium* by means of diarrhoea. These studies would provide useful data for risk assessments that consider illness as an outcome. The illness outcome is essential for risk assessment as the results obtained by risk assessments are usually compared and validated with epidemiological studies that are based on illness, not infection.

CONCLUSIONS

When assessing *Cryptosporidium* infection by means of oocyst shedding, this study suggests that within the 'experimental species' variable, only dogs, cats, rabbits and birds might not be appropriate to assess infection by means of oocyst shedding as these animal species appear to be more resistant to infection than mice and, therefore, less likely to shed oocysts following infection. In contrast, 'mice', 'other rodents', 'ruminants' and 'piglets' were all similarly likely to shed oocysts, and their use in dose–response studies when assessing infection by means of oocyst shedding is recommended.

When considering whether to use immunosuppressed or non-immunosuppressed experimental hosts to assess infection by means of oocyst shedding,

the multivariable analysis results suggest that it would be more appropriate to use immunosuppressed animals. Animals that are immunosuppressed are more likely to shed oocysts and probably more susceptible to infection, than non-immunosuppressed animals.

Furthermore, when designing experimental infectivity studies in which the infectivity is measured by means of the presence of oocyst shedding, it is also advisable to consider 'age' and 'oocyst dose' together. Differences by age were greater when the dose administered was larger.

Moreover, the results also suggest that it is beneficial to subject the oocysts to animal passage before inoculation into the experimental host when assessing infection by means of the presence of oocyst shedding in experimentally infected animals to increase the likelihood of oocyst shedding.

In this meta-analysis, the assessment of publication bias and heterogeneity for the oocyst shedding outcome indicate that it is important to publish both studies that have negative or non-significant results as well as those that have significant or positive results.

When presence of diarrhoea is the outcome in experimental studies, the results of the bivariate analysis suggest that the variables 'genotype', 'isolate source', 'animal passage', viability method', 'experimental species', 'age', 'immunosuppression', 'immunosuppression method', and 'administration route' should be considered when designing experimental studies as they are statistically associated with the presence of diarrhoea. Unfortunately, not enough studies have been performed for the diarrhoea outcome to ascertain the joint effects of the identified covariates. Thus, based on the results of this meta-analysis, it is crucial that more studies for diarrhoea should be conducted.

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DECLARATION OF INTEREST

None.

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