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Invited Review

In vitro culture systems for the study of apicomplexan parasites in farm animals

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ABSTRACT

In vitro culture systems represent powerful tools for the study of apicomplexan parasites such as *Cryptosporidium*, *Eimeria*, *Sarcocystis*, *Neospora*, *Toxoplasma*, *Besnoitia*, *Babesia* and *Theileria*, all with high relevance for farm animals. Proliferative stages of these parasites have been cultured in vitro employing a large variety of cell culture and explant approaches. For some, such as *Cryptosporidium* and *Eimeria*, the sexual development has been reproduced in cell cultures, while for others, animal experimentation is required to fulfill the life cycle. In vitro cultures have paved the way to exploit the basic biology of these organisms, and had a major impact on the development of tools for diagnostic purposes. With the aid of in vitro cultivation, studies on host–parasite interactions, on factors involved in innate resistance, stage conversion and differentiation, genetics and transfection technology, vaccine candidates and drug effectiveness could be carried out. The use of transgenic parasites has facilitated high-throughput screening of anti-microbial compounds that are active against the proliferative stages. Here, we review the basic features of cell culture-based in vitro systems for apicomplexan parasites that are relevant for farm animals, and discuss their applications with a focus on drug identification and studies of stage differentiation.

1. Introduction

Apicomplexan parasites are responsible for a variety of diseases in humans, pets and/or farm animals, and are thus of considerable medical and economic importance. Those most relevant for farm animals are *Babesia*, *Besnoitia*, *Cryptosporidium*, *Eimeria*, *Neospora*, *Sarcocystis*, *Theileria* and *Toxoplasma*, causing diseases of great socio-economic impact worldwide. Treatment options for many of these diseases are limited and include either culling of infected livestock, prevention of infection by vaccination and/or chemotherapy. Chemotherapy would certainly be a viable choice, provided potent and safe drugs are made available that are inexpensively produced, easy to apply and that preferentially interfere in more than just one stage of the life cycle by exhibiting selective anti-parasitic toxicity. Thus, knowledge on suitable targets for intervention must be generated and candidate compounds must be thoroughly characterised with regard to their mechanisms of action. For such studies, in vitro culture systems have been increasingly applied. The pathogenesis of the parasites mentioned above is related to intracellular stages of their life cycle. In order to investigate the molecular features of these stages and the mechanisms that lead to differentiation or stage conversion, animal experimentation has been combined with, or even replaced by, suitable in vitro culture systems, which employ primary mammalian cells or cell lines of various origins as host and/or feeder cells. In this review, we will document the development of culture methods for apicomplexan parasites that are relevant for farm animals, and highlight the advantages and limitations of these in vitro systems.

2. In vitro culture techniques

2.1. Cryptosporidium spp.

Cryptosporidiosis, an important opportunistic infection in immune-compromised humans, is characterised by persistent diarrhoea and is also found in many young farm mammals including lambs, calves, goat kids and piglets, as well as cats and dogs, with sometimes fatal consequences. Infection in farm and/or domestic animals may be a reservoir for infection in susceptible humans. The disease is caused by a variety of *Cryptosporidium* spp., which are regarded as a distantly related lineage that is in fact not coccidian, but occupies many of the same ecological niches (Barta and Thompson, 2006). *Cryptosporidium* isolates from different regions express different antigens and exhibit different degrees of virulence, infectivity and sensitivity to drugs (Fayer et al., 2000; Perez Cordón et al., 2007). The parasites commonly act in concert with other enteropathogens to produce intestinal injury and diarrhoea, and the disease is self-limiting in immunocompetent humans and older animals.

Cultures of *Cryptosporidium* spp. are initiated by excystation of oocysts and seeding of sporozoites onto host cells of various

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origins, where they typically form parasitophorous vacuoles, which are intracellular but located at the very peripheral part of the host cell surface, that form an intracellular but extra-cytoplasmatic compartment. The appearance of extracellular stages of *Cryptosporidium parvum* in vitro was first described in Madin-Darbey canine kidney (MDCK) cells (Rosales et al., 1993). A comparison of 24 isolates from different origins that were cultured on a variety of cell types confirmed that MDCK cells were best suited for supporting parasite proliferation (Siripanth et al., 2004). The complete development of *C. parvum* up to oocyst development was reported in bovine fallopian tube epithelial (BFTE) cells (Yang et al., 1996), human Caco-2, HT29 and HCT-8 cell lines (Maillot et al., 1997; Hijjawi et al., 2001, 2002; Borowski et al., 2010) and in primary cultures of rabbit auricular chondrocyte (VELI) cells (Lacharme et al., 2004). Recently, chick embryo tracheal organs were used to culture oocysts and sporozoites of *Cryptosporidium baileyi*, which infects the respiratory tract (Zhang et al., 2012).

Moreover, the completion of the life cycle of *C. parvum* (cattle genotype) was reported in RPMI-1640 medium devoid of host cells, yielding culture-derived oocysts infective for mice (Hijjawi et al., 2004). However, this phenomenon seemed to be restricted to the *C. parvum* cattle strain and could not be reproduced, neither with *C. parvum* Moredun or IOWA strains nor with *Cryptosporidium hominis* strain TU502 (Girouard et al., 2004) and is therefore still controversial (Karanis and Aldeyarbi, 2011).

Optimisation of in vitro culture conditions also included studies on the viability of sporozoites immediately following excystation from oocysts. Electron microscopy and labelling with fluorescent dyes showed that post-excysted sporozoites underwent morphological changes and experienced a rapid loss of viability (Matsubayashi et al., 2010). Thus, King et al. (2011) more recently optimised procedures for excystation and synchronicity of infection of host cells in order to eliminate low levels of infectivity, delayed life cycle development and poor synchronicity, which are the main factors that hinder appropriate investigations, e.g. on drug efficacy. Attempts to modify in vitro culture conditions also included the use of serum-free media for in vitro propagation of *Cryptosporidium* (Woods and Upton, 2007), thus eliminating another important variable from the experimental system. Furthermore, a three-dimensional enteric cell culture model may be suitable for in vitro studies on *Cryptosporidium* spp. (Mathis, 2007).

In order to improve the identification of stages of *C. parvum* in culture, a novel approach combining scanning electron microscopy with immunolabelling was introduced (Edwards et al., 2012). This method correlated high-resolution microstructural information with defined *Cryptosporidium* stages and allowed identification and visualisation of microgametocyte development in cell culture.

Recent developments such as the establishment of transient transfection techniques for *C. parvum* sporozoites (Li et al., 2009), and sequencing of the genomes of *C. parvum*, *C. hominis*, and *Cryptosporidium muris* have uncovered a plethora of unique features of this genus, which can now be further exploited to understand the basic biology and epidemiology of this parasite (Jex et al., 2011; Widmer and Sullivan, 2012).

2.2. Eimeria spp.

The genus *Eimeria* is comprised of approximatively 1,700 described species which infect chicken, cattle, sheep, rabbits, rodents, fish, seals, cats, dogs and others. Coccidiosis is an opportunistic infection, which is present most commonly in animals housed or confined in small areas under stressful conditions and infections occurs via ingestion of sporulated oocysts. All *Eimeria* spp. that infect mammals do this in a host-specific manner, with few exceptions. *Eimeria* infection causes intestinal epithelium necrotisation and bloody diarrhoea, often with fatal consequences, due to large numbers of oocysts and merozoites released from intestinal cells. In some instances, such as in rabbits, the liver can also be affected. The infections with *Eimeria* in immunocompetent hosts are usually self-limiting and end spontaneously within a few weeks unless reinfection occurs. For chemotherapeutical treatment, a wide range of drug classes is available. There are, however, increasing problems with drug resistance amongst many coccidian species, thus periodic rotation between different drug groups and the use of combination (cocktail) drugs to minimise the occurrence of resistance are recommended (Tewari and Maharana, 2011).

Many *Eimeria* spp. have been maintained as merozoites and schizonts in primary intestinal epithelium cell cultures (Augustine, 2001). Oocyst development has been observed, but to complete the life cycle and propagate the parasites, infection experiments in the appropriate target host are performed using cell culture-derived parasites. Earlier studies, for e.g. using *Eimeria bovis*, have shown that several cell types sustain in vitro development to varying degrees, such as bovine, human and porcine endothelial cell lines, bovine foetal gastrointestinal cells (BFGC), bovine umbilical vein cells (BUVEC), bovine spleen lymphatic endothelial cells, MDBK cells and African green monkey kidney epithelial (Vero) cells (Hermosilla et al., 2002). Sporozoite invasion of host cells was completed within the first 12 h following excystation, and within the next 12 h new host cells were invaded. While further endogenous development took place in bovine cells, parasites survived for a minimum of 3 weeks in all cells investigated. However, depending on the host cells used, *E. bovis* development mimicked more closely the in vivo situation in bovine endothelial cell lines, exhibited increased proliferation of first generation merozoites in BFGCs, or developed enlarged parasitophorous vacuoles in Vero cells. Studies on comparative development, but on *Eimeria tenella*, showed that many different cell lines seemed to support development of *E. tenella*, but MDBK cells appeared to be best suited (Tierney and Mulcahy, 2003).

Transient transfection of *E. tenella* was first established in 1998 with transfected sporozoites forming first generation schizonts in vitro (Kelleher and Tomley, 1998). The path to stable transfection was opened by restriction-mediated transfection (Liu et al., 2008). Parasites transfected with plasmids carrying yellow fluorescent protein (YFP) under control of a constitutive promotor could complete their entire endogenous development in cell culture. Fluorescent sporozoites were demonstrated after transfection of *E. tenella* sporozoites and following culture in primary chicken kidney cells, fluorescent schizogony and gametogony stages were observed, and fluorescent oocysts were seen between 200 and 327 h p.i. (Shi et al., 2008). As a consequence, stable transfection was established as a powerful tool for reverse genetic studies not only in *E. tenella*, but also in other *Eimeria* spp. (Clark et al., 2008; Kurth and Entzeroth, 2009; Yan et al., 2009; Hanig et al., 2012).

2.3. Sarcocystis spp. and (Cysto)isospora spp.

The genus *Sarcocystis* is comprised of approximately 130 recognised species infecting cattle, sheep, pigs and horses (Tenter, 1995). *Sarcocystis* spp. undergo a two-host life cycle. Within their intermediate hosts, bradyzoite-stage parasites are often located in muscular tissue cysts, but in some instances the parasites can also cross the blood–brain barrier. In horses, which act as an aberrant host for *Sarcocystis neurona*, infection leads to equine protozoal myeloencephalitis (EPM), causing severe neurological symptoms. EPM is widespread and common in the United States of America (USA) (MacKay, 1997). Treatment is rarely required, except in cases where e.g. the CNS is involved. Infection does not improve, however, if the CNS damage has occurred before treatment begins (Dubey et al., 2001). Due to the lack of clinical symptoms in most cases, *Sarcocystis* infection in humans is most likely severely