Veterinary Parasitology 225 (2016) 91–98

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Free-living protozoa in the gastrointestinal tract and feces of pigs: Exploration of an unknown world and towards a protocol for the recovery of free-living protozoa

N. Chavatte[a], E. Lambrecht[a], I. Van Damme[a], K. Sabbe[b], K. Houf[a,*]

[a] Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium
[b] Department of Biology, Faculty of Sciences, Ghent University, Krijgslaan 281, 9000 Ghent, Belgium

ARTICLE INFO

Article history:
Received 8 January 2016
Received in revised form 1 June 2016
Accepted 2 June 2016

Keywords:
Free-living protozoa
Free-living amoebae
Gastrointestinal tract
Feces
Pigs

ABSTRACT

Associations with free-living protozoa (FLP) have been implicated in the persistence of foodborne pathogenic bacteria in food-related environments. To date however no information is available on the presence of FLP in the gastrointestinal tract (GIT) of pigs, which represents an important reservoir for zoonotic foodborne bacteria and hence a potential location for associations with FLP. This is at least partly due to the lack of adequate protocols to recover FLP from intestinal content and feces. In the present study different protocols to recover FLP from the porcine GIT and feces were tested. The most effective protocols were then applied to explore the presence of live FLP in the pig GIT and feces. A filtration based protocol was identified as the most suitable method to recover viable FLP from the porcine GIT and feces. Cultivable FLP were recovered from different parts of the GIT, suggesting at least a transient presence of FLP in this habitat. Free-living amoebae species (*Acanthamoeba* spp., *Hyperamoeba* sp., *Vannella* sp., *Vermamoeba vermiformis,* hartmannellids and vahlkampfiids) but also ciliates (*Colpoda* sp. and *Tetrahymena/Glaucoma* lookalike) and flagellates (cercomonads, bodonids and glissomonads) were recovered and cultured from pig intestinal content. *Acanthamoeba hatchetti* and *Filamoeba sinensis* were isolated for the first time from pig intestinal content. Despite high gastric acidity, non-cyst forming amoeba species were also detected which suggests survival of their trophozoites in the animal GIT.

1. Introduction

Studies dealing with intestinal protozoa of mammals are mainly focused on parasitic protozoa like *Giardia*, *Cryptosporidium, Entamoeba* and *Toxoplasma* (Geurden et al., 2008; Hamnes et al., 2007; Robertson et al., 2010; Solaymani-Mohammadi and Petri, 2006). Notwithstanding their importance as causative agents of disease in both animals and humans, virtually nothing is known about the presence of free-living protozoa (FLP) in the gastrointestinal tract and feces of mammals.

Free-living protozoa, *i.e.* unicellular heterotrophic eukaryotes which do not have an obligate parasitic life cycle, are widespread in aquatic and soil habitats. Recent research has revealed that they can play an important role in the transmission and persistence of foodborne pathogenic bacteria in different environments (Vaerewijck et al., 2014). While FLP and bacteria typically show a predator-prey interaction, some bacteria resist protozoan uptake and digestion and are able to survive and even grow inside FLP (Matz and Kjelleberg, 2005). Free-living protozoa can thus act as a shelter and protect intracellular bacteria against harsh environmental conditions (King et al., 1988; Lambrecht et al., 2015). Moreover, FLP are so called Trojan horses, enabling internalized bacteria to pass the first line mammal immune system (Barker and Brown, 1994; Greub and Raoult, 2004).

Though most FLP are harmless for mammals, some taxa are opportunistic pathogens, such as the well-studied free-living amoeba (FLA) genera *Acanthamoeba, Naegleria* and *Balamuthia.* Information about the presence of FLP in mammals is limited to a few studies and reports on FLA recovered from stool and feces (see below). More than 80% of healthy human individuals have been tested seropositive for antibodies against *Acanthamoeba polyphaga* (Chappell et al., 2001), indicating that FLA are more common than suspected. *Acanthamoeba* spp. and other emerging opportunistic pathogenic FLA species like *Hartmannella* sp., *Hyperamoeba* sp. and *Vahlkampfia* sp. have been recovered from human stool of both

* Corresponding author.
E-mail addresses: natascha.chavatte@ugent.be (N. Chavatte), ellen.lambrecht@ugent.be (E. Lambrecht), inge.vandamme@ugent.be (I. Van Damme), koen.sabbe@ugent.be (K. Sabbe), kurt.houf@ugent.be (K. Houf).

http://dx.doi.org/10.1016/j.vetpar.2016.06.002
0304-4017/

healthy and infected individuals (Bradbury and Forbes, 2013; de Moura et al., 1985; Mergeryan, 1991; Zaman et al., 1999a, 1999b). *Acanthamoeba* strains belonging to the most pathogenic genotype T4, have also been isolated from cattle and squirrel feces (Lorenzo-Morales et al., 2007; Niyyati et al., 2009).

Pigs have a similar microbiome as humans and are susceptible to the same (though not all) enteric pathogens (Zhang et al., 2013). The gastrointestinal tract of pigs is also an important reservoir for zoonotic foodborne bacteria like *Salmonella enterica* Typhimurium (Mikkelsen et al., 2004), *Yersinia enterocolitica* (Van Damme et al., 2015), *Campylobacter* spp. (Aguilar et al., 2014; Madden et al., 2007), verotoxigenic *Escherichia coli* (VTEC) (Dixit et al., 2004; Mainil and Daube, 2005), and *Arcobacter* spp. (Van Driessche et al., 2004). With pork being one of the most frequently consumed kinds of meat (Devine, 2003), control of foodborne pathogens in the pig reservoir is important for public health. As pigs are omnivorous and likely to be exposed to environmental FLP, the gastrointestinal tract creates an ideal niche and meeting place for foodborne pathogens and FLP, favouring the close association between both microbial groups.

To date, virtually nothing is known about the presence of viable FLP in the porcine gastrointestinal tract and their excretion in the feces. Partly due to lack of protocols to recover FLP from intestinal content, thorough research of this microbial group in this habitat is impeded. The hypothesis of the present study is that besides the ubiquitous dispersal of FLP in the environment, FLP are also present and passing through the gastrointestinal tract of pigs and consequently, due to their co-occurrence with foodborne pathogens in this environment, are potentially important for the persistence and transmission of pathogenic bacteria in this habitat.

The aim of the present study is to explore the presence of FLP in the pig gastrointestinal tract and feces. However, as standardized protocols implemented in parasitic research, are not yet applied to recover FLP from intestinal content, a suitable protocol to recover FLP from the porcine gastrointestinal tract and feces is developed and tested.

2. Material and methods

2.1. Development and testing of protocols for the recovery of FLP from the porcine gastrointestinal tract and feces

A comprehensive review of literature was conducted in search of existing protocols. Methods commonly applied in parasitic research for the examination of stool and feces samples and methods implemented for the recovery of FLA from water and soil samples were selected as a starting point for the development of a suitable recovery protocol for FLP from pig intestinal content. Four methodologies, newly developed or adapted and optimized where necessary, were tested on artificially inoculated fecal samples: (1) a filtration based design; (2) a sedimentation based design; (3) Ludox and magnesium sulphate density gradient centrifugation (based on Heip et al. (1985) and Dryden et al. (2005)); and (4) formalin-ethyl acetate sedimentation concentration (modified from a protocol by Garcia (2007) for *ova* and parasite evaluation in stool specimens).

Three FLP organisms, representing the three main morphogroups, were selected for the artificial inoculation: *Tetrahymena pyriformis* (ciliate, CCAP 1630/1W), *Acanthamoeba castellanii* (amoeba, ATCC 30324) and *Cercomonas* sp. (flagellate, environmental isolate from a meat-cutting plant, 2008). Cultivation of FLP was performed as previously described by Chavatte et al. (2014).

Three fecal samples (originating from different pigs) were aseptically collected during defecation, individually packed in plastic containers, transported to the lab and processed the same day. A minimum of 10 g of feces was put in a sterile Petri dish followed by artificial inoculation with 2 ml of either a single species or a mixed species cell suspension (*T. pyriformis, A. castellanii* and *Cercomonas* sp.). The final inoculation concentration of the model organisms was 10^5 cells/ml for *T. pyriformis* and *A. castellanii* and 10^4 cells/ml for *Cercomonas* sp. for the single species suspension, and 10^5 cells/ml (*1:1:1*) for the mixed species cell suspension.

Inoculated samples were stored for 1 h at room temperature. Samples were then transferred to a stomacher bag (with filter), Page's Amoeba Saline (PAS, CCAP recipe) was added to a final weight of 100 g and samples were homogenized for 2 min. Subsamples of the homogenate were immediately used for the filtration and sedimentation protocols listed below. Non-inoculated samples were included as controls.

2.1.1. Filtration

Filtration of samples over small pore size filters (1.5 μm) is a commonly used technique to recover FLA from water and soil samples (Pernin et al., 1998; Rahdar et al., 2012). The filtration based adapted protocol included three filtration steps. The first two exclude fecal debris, the final intercepts FLP cells, allowing bacterial cells to pass through.

50 ml of the homogenate was filtered consecutively over a 0.5 mm woven wire mesh filter (VWR, Belgium), a 200 μm polyethylenterephthalat cell strainer (PluriSelect, HiSS Diagnostics, Germany) and a 1.6 μm glass microfiber filter (VWR, Belgium). The 1.6 μm filter was then put in a 50 ml tube filled with 20 ml PAS and placed on a lab shaker for 5 min at 350 rpm. The suspension and filter were transferred to a Petri dish and autoclaved dry rice grains were added as a nutrient source. Sealed Petri dishes were incubated at 25 °C and 7 °C. Samples were examined daily (inverted microscopy, magnification ×400) for the presence of FLP. The samples were washed on a regular basis and sub cultured (*1:10*) with PAS.

2.1.2. Sedimentation

The sedimentation protocol is based on the observation that amoeba sink to the bottom of a conical tube, while motile ciliates and flagellates remain in the supernatant. Further cultivation is done by means of inoculation on Non-Nutrient Agar (NNA) plates for amoebae and in liquid culture for ciliates and flagellates. Inoculation on NNA plates seeded with heat-killed *Escherichia coli* is a culturing method to recover FLA from water and soil, proposed by Page (1988).

50 ml of the homogenate was collected in a 50 ml polypropylene conical tube (Novolab, Belgium) and incubated for 2 h at room temperature. 20 ml of the supernatant was removed and transferred to a Petri dish with addition of sterile dry rice grains. Sealed Petri dishes were incubated at 25 °C and 7 °C. In addition, 30 μl of the sediment was streaked onto NNA plates seeded with *E. coli* and incubated at 25 °C. Samples were examined daily (inverted microscopy, magnification ×400) for the presence of FLP. The samples were washed on a regular basis and sub cultured (*1:10*) with PAS.

2.1.3. Ludox and magnesium sulphate density gradient centrifugation

Density gradient centrifugation or flotation techniques are commonly used to recover parasite eggs and oocysts from stool or feces (Dryden et al., 2005). Furthermore, it is applied to extract meiofauna from marine sediments (Heip et al., 1985) and ciliates from coastal soils (Zhao et al., 2012). Based on the protocols described by Heip et al. (1985) and Dryden et al. (2005), 10 g of artificially inoculated feces was used to test a density gradient centrifugation protocol with Ludox or magnesium sulphate ($MgSO_4$) as flotation medium. Ludox (a colloidal silica polymer, specific gravity 1.18 g/cm^3) or $MgSO_4$ (specific gravity 1.28 g/cm^3) was added (solution) to the fecal samples (final volume 250 ml) and gently mixed prior to cen-