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Free-living Amoebae May Serve as Hosts for the *Chlamydia*-like Bacterium *Waddlia chondrophila* Isolated from an Aborted Bovine Foetus

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Summary. *Chlamydia*-like endocytobionts are commonly observed in protozoan hosts. Therefore, we examined the potential of 21 different species of free-living amoebas to serve as hosts of a newly found bacterium, *Waddlia chondrophila.* The *Chlamydia*-like bacterium *Waddlia chondrophila* 2032/99 was originally isolated from an aborted bovine foetus in Rheinstetten, Germany. The inoculum of the obligate intracellular agent was prepared from Buffalo Green Monkey (BGM) cells. The infection of *Hartmannella vermiformis* OS101 revealed typical morphological stages of a *Chlamydia*-like life cycle, including the presence of elementary and reticulate bodies. The following infection studies with a *Hartmannella*-adapted *Waddlia* isolate showed that also *Acanthamoeba* sp. Gr. II HLA, *Vahlkampfia ovis* Rhodos, *H. vermiformis* C3/8, *Hyperamoeba*-like *amoeba* B1,2-100PE and *Dictyostelium discoideum* Sö-P2 supported the growth of *Waddlia.* An interesting finding was that *Hartmannella*-adapted *Waddlia* exhibited a broader host range when compared to the BGM cell isolate. The concept that *Waddlia* may fall into the group of environmentally preadapted pathogens is discussed.

Key words: *Acanthamoeba, Chlamydia,* endoparasite, *Hartmannella,* host spectrum, *Hyperamoeba, Neochlamydia, Simkania,* ultrastructure, *Waddlia chondrophila.*

INTRODUCTION

Chlamydiae are important obligate intracellular pathogens. They are known to be the causative agents of a variety of diseases including infections of the eye, and the respiratory and genital tracts. The life cycle of Chlamydiae is characterized by the development of reticulate bodies, which divide intracellularly by binary fission, and the elementary bodies, which are specialized for transmission (Stephens 1999).

The recent isolation of several novel *Chlamydia*-related bacteria from free-living amoebae, contaminated cell culture and an aborted bovine foetus led to the reclassification of the Chlamydiales and the establishment of the families Parachlamydiaceae, Simkaniaceae, and Waddliaceae (Everett *et al*. 1999, Poppert *et al.* 2002). The clinical significance of the so-called environmental chlamydiae is still unclear. This is also true for *Waddlia chondrophila*, which was isolated from the lung and liver of an aborted bovine foetus (Dilbeck *et al*. 1990, Rurangirwa *et al*. 1999). A second isolation of this species from an aborted bovine foetus in

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Germany suggests that it may be associated with abortions in cattle (Henning *et al*. 2002). However, since this second case was associated with Neosporosis the direct influence of *W. chondrophila* on reproductive failure remains to be established.

Within the Chlamydiales it was shown that the Parachlamydiaceae naturally infect amoebae (Everett *et al*. 1999). In addition we previously isolated the *Chlamydia*-like strains Bn9 from *Acanthamoeba* sp. (Michel *et al*. 1994) and A1Hsp1 from *Hartmannella vermiformis* that were later described as novel species *Parachlamydia acanthamoebae* and *Neochlamydia hartmannellae* respectively (Amann *et al*. 1997, Horn *et al*. 2002). For *Acanthamoeba* spp., approximately one of five isolates from both environmental and medical samples contains bacterial endocytobionts (Fritsche *et al.* 1993). In addition it has been shown that *Chlamydia*-like endosymbionts are able to enhance the amoebic cytopathogenicity *in vitro* which may have clinical significance (Fritsche *et al*. 1998).

In this study we evaluated the amoebae host spectrum of the German *W. chondrophila* isolate 2032/99. The findings presented here may help to identify the environmental reservoir and to analyze the epidemiological importance of this organism.

MATERIALS AND METHODS

Isolation and maintenance of *Waddlia chondrophila*. *Waddlia chondrophila* strain 2032/99 was isolated from a bovine fetus, which was aborted on day 228 of gestation. Buffalo Green Monkey (BGM) cells were used for the isolation and cultivation of the bacteria. The cell culture medium was composed of RPMI 1640 and PFEK-1 medium (Biochrom, Berlin, Germany) (1 : 1/v : v) containing 20 mmol HEPES, 5% new-born calf serum, 50 µg/ml gentamycin, 100 µg/ml vancomycin, 2.5 µg/ml amphotericin B, 1% vitamins, 1% amino acids, and 2% glutamine (Henning *et al.* 2002). Infected BGM cells were incubated at 37°C. The cultures were examined for inclusions by phase-contrast microscopy or by staining according to the method of Giménez (1964).

Protozoan strains and culture. The protozoa used in this study are listed in Tables 1 and 2. *Naegleria gruberi* (CCAP 1518/1e) and *N. lovaniensis* (Aq/9/45D) were kindly provided by Johan De Jonckheere, the *Balamuthia mandrillaris* strain CDC: VO39 was provided by Klaus Janitschke. The *Acanthamoeba* strain HLA was isolated from the cornea of a keratitis patient and has been provided by Horst Aspöck. The *Dictyostelium* strain Sö-P2 was isolated from soil. The protozoan cultures were maintained on NN-agar seeded with *Enterobacter cloacae* according to Page *et al*. (1988). *N. gruberi*, *N. lovaniensis*, and *Hartmannella vermiformis* (OS 101) were grown axenically on SCGYE-medium according to De Jonckheere (1977).

Growth of bacteria within protozoa. The cell suspension of a 6-8 days old infected BGM culture was passed through a membrane filter with a pore size of 1.2 µm. Alternatively, the bacterial inoculum was prepared from infected *H. vermiformis* OS101 by freeze-thawing and passage through a 1.2 µm filter. Bacterial elementary bodies from 100 ml filtrate and trophozoites of log-phase protozoa were coincubated on NN-agar or in SCGYE-medium. After 48 h the infected cell cultures were inspected daily for a time period of 10 days. Morphological alterations and symptoms of infection were compared with non-infected host cells.

Electron microscopy. Infected host cells were harvested after 5 days post infection. The cell suspension was centrifuged for 10 min at 600 g. The resulting pellet was fixed in 3% glutaraldehyde (1 h), transferred to 0.1M cacodylate buffer, postfixed in 1% osmium tetroxide and embedded in Spurr resin. Sections were stained with uranyl acetate and Reynold's lead citrate and examined using a Leo EM 910 electron microscope.

RESULTS

Cocultivation of *W. chondrophila* and protozoa. To evaluate whether *Acanthamoeba* sp., *H. vermiformis* OS 101, *N. gruberi* and *N. lovaniensis* are suitable hosts for *W. chondrophila* isolate 2032/99, we analyzed the intracellular multiplication of the bacteria by light microscopy, phase contrast microscopy and transmission electron microscopy. The bacterial inocula were prepared from infected BGM cells and the results of the coincubations on NN-agar plates and in axenic SCGYE-medium are summarized in Table 1. After 5 days of cocultivation *H. vermiformis* OS101 showed characteristic signs of infection including glassy appearance, prevention of cell migration and the presence of intracellular coccoid bacteria. These features resemble previous observations of *Neochlamydia* infected host cells (Horn *et al*. 2000). Subsequent subculture showed that the association of *Waddlia* and its experimental host *H. vermiformis* was stable. *Acanthamoeba* sp. Gr II, *N. gruberi* and *N. lovaniensis* were not susceptible to infection with *Waddlia*. The inspection of the *Naegleria* Nbeck led to the detection of intracellular *Waddlia*. However the cocci were regularly lost after the subculture of the host.

Transmission electron microscopy of *W. chondrophila* infected *Hartmannella* trophozoites revealed that numerous coccoid bacteria were located either within the cytoplasm or within membrane bound vacuoles (Fig. 1). At higher magnification typical morphological characteristics of the Chlamydiales were observed and the two developmental stages could be visualized simul-

Table 1. Cocultivation of free-living amoebae with *Waddlia chondrophila* isolated from BGM cell culture.

Host amoeba	Source	Intracellular replication
NN-agar plates[1]		
Acanthamoeba sp. Gr. II C3 ATCC50739	Potable water reservoir	-
Acanthamoeba sp. Gr. II HLA	Keratitis patient	-
Naegleria sp. Nbeck	Aquarium	(+)[3]
Hartmannella vermiformis OS101	Hospital tap water	+++
SCGYE medium (axenic)[1]		
Naegleria gruberi CCAP:1518/1e	Unknown	-
Naegleria lovaniensis Aq/9/1/45D	Aquarium	-
Hartmannella vermiformis OS101	Hospital tap water	++

[1] Infection medium. [2] Intracellular replication of *W. chondrophila* was determined by microscopic inspection. [3] Intracellular bacteria were regularly lost after subculture of the host.

Table 2. Cocultivation of protozoa with *Waddlia chondrophila* isolated from *Hartmannella vermiformis* OS101.

Host protozoa	Source	Intracellular replication[1]
Acanthamoeba sp. Gr. II C_3 ATCC50739	Water reservoir	-
Acanthamoeba lenticulata Gr. III 89	Nasal mucosa	-
Acanthamoeba lenticulata Gr. III 45, ATCC50703	Nasal mucosa	-
Acanthamoeba lenticulata Gr.III 72, ATCC50704	Nasal mucosa	-
Acanthamoeba sp., Gr. II HLA	Keratitis patient	+++
Acanthamoeba comandoni Gr. I Pb30/40	Greenhouse	-
Naegleria gruberi CCAP:1518/1e	Unknown	+[2]
Naegleria gruberi Nbeck	Aquarium	-
Naegleria lovaniensis Aq/9/1/45D	Aquarium	-
Vahlkampfia ovis Rhodos	Puddle/Rhodos	+++[3]
Willaertia magna A1,2PbFl2	Greenhouse	-
Willaertia magna NI4Cl1	Pond/India	-
Hartmannella vermiformis OS 101	Hospital water	+++
Hartmannella vermiformis C3/8	Water reservoir	+++
Echinamoeba sp. PVC/Mühlh.	Potable water	-
Vannella miroides DentG1	Dental unit	-
Comandonia operculata WBT	Water reservoir	-
Balamuthia mandrillaris CDC:Vo39	*Papio sphinx*	-
Hyperamoeba-like amoeboflagellate B1,2-100PE	Water reservoir	+++
Dictyostelium discoideum Berg 25	Nasal mucosa	+[2]
Dictyostelium discoideum Sö-P2	Soil, Würzburg	+++

[1] Intracellular replication of *W. chondrophila* was determined by microscopic inspection.[2] Intracellular bacteria were regularly lost after subculture of the host.[3] Infected cultures were able to produce cysts.

taneously. The highly condensed coccoid stages (approximately 0.4 µm) were identified as elementary bodies (Fig. 2). The thin walled particles (approximately 0.6 µm) some of which show binary fission were identified as reticulate stages. These results show that *W. chondrophila* can infect and develop within *H. vermiformis*.

Infection of different protozoa species by *Hartmannella*-adapted *Waddlia*. In order to determine the host range of *Waddlia*, 21 different protozoan

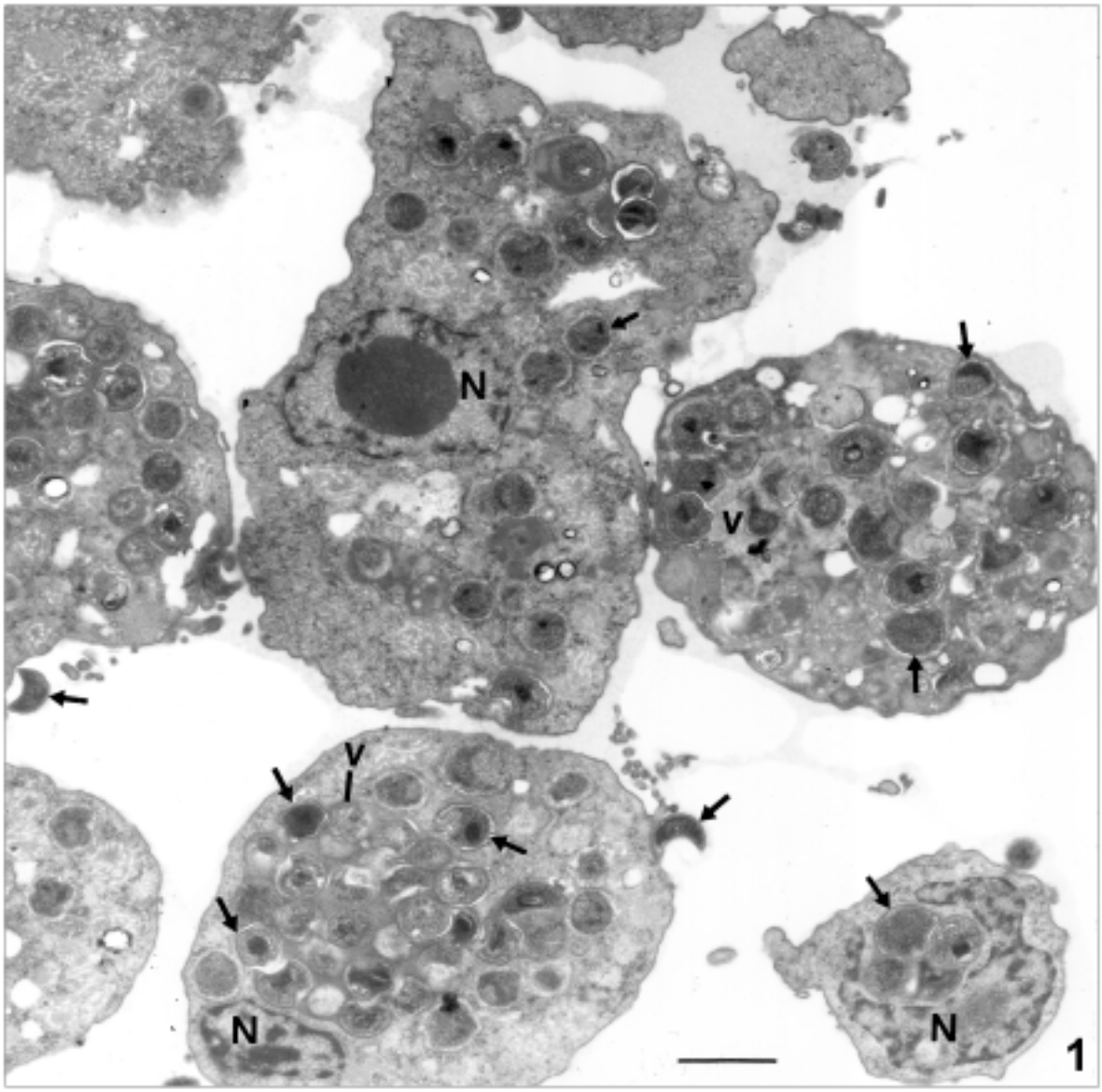

Fig. 1. Transmission electron micrograph of intracellular *Waddlia chondrophila* 2032/99 within *Hartmannella vermiformis* OS101. The protozoan trophozoites harbour numerous coccoid endocytobionts (arrows). The bacteria are located either within the cytoplasm or within vacuoles (v). N - nucleus of the host amoeba. Scale bar 1.0 µm.

species belonging to the genera *Acanthamoeba*, *Naegleria*, *Hartmannella*, *Vahlkampfia*, *Willaertia*, *Echinamoeba*, *Vannella*, *Comandonia*, *Balamuthia*, the *Hyperamoeba*-like amoeba and *Dictyostelium* were tested in cocultivation assays. The bacterial inoculum was prepared after the cultivation of *Waddlia* in *H. vermiformis*. After five transfers of infected hartmannellae into fresh axenic medium the trophozoites were submitted to freeze-thawing and subsequent filtering through a millipore filter. The filtrate with *Hartmannella*-adapted endocytobionts was then added to the different protozoan species on NN-agar. The successful infection of *H. vermiformis* strain OS101 was used as positive control.

Daily monitoring by microscopy showed that *Acanthamoeba* sp. Gr. II, HLA, *V. ovis* Rhodos, *H. vermiformis* C3/8, the *Hyperamoeba*-like amoeba, and *D. discoideum* Sö-P2 harboured numerous replicating *Waddlia* endocytobionts (Table 2). The infection by endocytobionts resulted in the inhibition of cyst formation in the free-living amoebae and fruiting body development in *Dictyostelium*, respectively. The only exception was

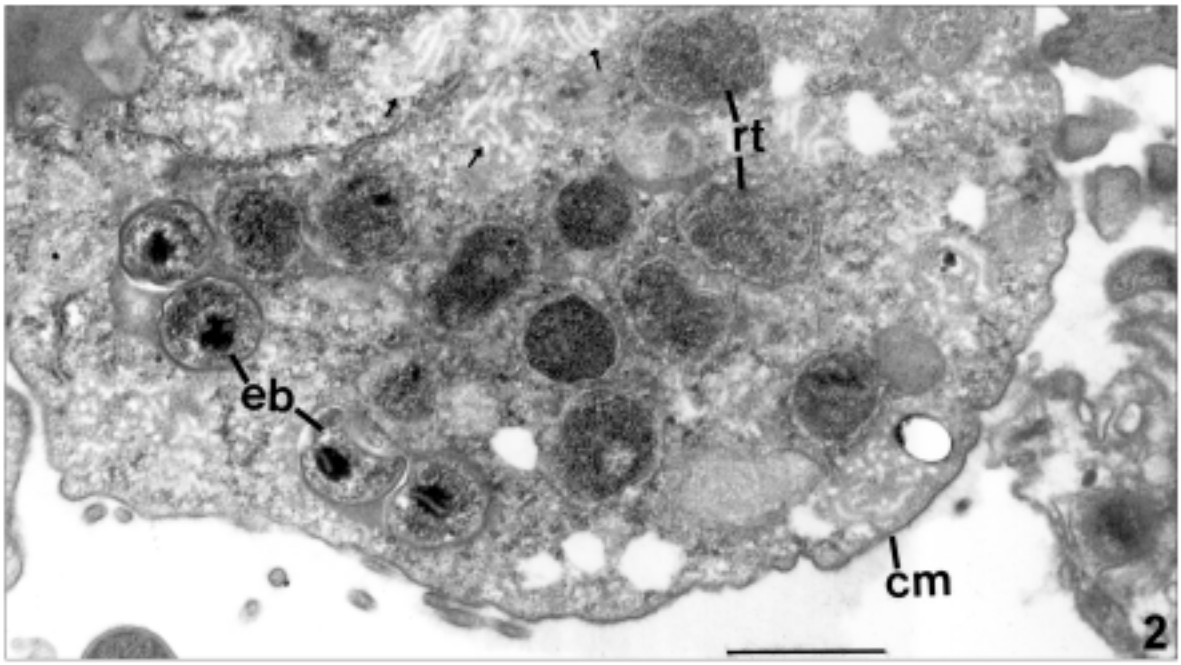

Fig. 2. Transmission electron micrograph of intracellular *Waddlia chondrophila* 2032/99 within *Hartmannella vermiformis* OS101. The detail of a sectioned *Hartmannella trophozoite* shows the differentiation of the endocytobiont into reticulate stages (rt) and elementary bodies (eb). Arrows indicate mitochondria. Cm - cell membrane of the trophozoite. Scale bar 1.0 µm.

found with *V. ovis* since infected cultures of this host were still able to differentiate into regular cysts. Very weak and transitory infections were observed with *N. gruberi* CCAP and *D. discoideum* Berg 25. Interestingly, a heavy infection was observed with *Acanthamoeba* sp. Gr. II HLA. This observation is remarkable since *Acanthamoeba* spp., Gr. II, HLA was originally resistant to infection by *Waddlia*. This result and the transitory infection of *N. gruberi* CCAP1518/1e suggest that the adaptation of *Waddlia* to *Hartmannella* results in a broader host spectrum of the endocytobiont.

DISCUSSION

Chlamydia-like endocytobionts are commonly observed in protozoa (Michel *et al.* 1994, Amann *et al*. 1997, Horn *et al*. 2000, Fritsche *et al*.2000, Michel *et al*. 2001). The ability to grow intracellularly within protozoa could have preadapted these bacteria as pathogens of higher eukaryotes (Corsaro *et al*. 2002). Accordingly it is likely that protozoa may also represent a reservoir for the chlamydia-like bacterium *W. chondrophila*. In order to evaluate the amoebae host spectrum we transferred this obligate intracellular endocytobiont from BGM cells into *H. vermiformis*. Detailed transmission electron microscopy of *Waddlia* infected *Hartmannella* cells revealed typical morphological stages of a *Chlamydia*-like life cycle, including the presence of elementary and reticulate bodies. The following coculture assays with 21 different protozoa species revealed that species of the genera *Acanthamoeba*, *Vahlkampfia*, *Hartmannella*, *Hyperamoeba* and *Dictyostelium* were able to support bacterial growth. The infected cells were killed and lysed by the replicating bacteria. However the amoebae generation time was obviously shorter than the period between infection and host cell lysis since infected cultures could easily be maintained.

The observed host range of *Waddlia* is unequally wider when compared to the *Chlamydia*-like *Hartmannella* endocytobiont *Neochlamydia hartmannellae* (Horn *et al.*, 2000). This previously analyzed strain was not able to propagate within closely related protozoa except the original host, two more *H. vermiformis* strains and *D. discoideum*. However, in contrast to *Waddlia*, which inhibited the formation of *Dictyostelium* fruiting bodies, *Neochlamydia* did not interfere with the underlying differentiation processes.

An interesting finding of this study was that *Hartmannella*-adapted *Waddlia* exhibited a broader

host range when compared to the BGM cell isolate. This suggests that *Waddlia* possesses an adaptive potential to broaden its host range. The aggressive behaviour of the endocytobionts within the tested protozoa and the ability to prevent cyst formation also strengthen the view of a limited evolutionary specialization of *Waddlia*. Another possible hypothesis is that *Waddlia* survives within a yet unrecognized reservoir. The best-adapted host identified so far appears to be *V. ovis*. This host exhibited high intracellular bacterial numbers and infected cells maintained their ability to differentiate into cysts.

Many protozoa are not very selective with respect to the uptake of bacteria (Görtz and Michel 2003). In evolutionary time frames this behaviour may help environmental bacteria to overcome host specificities. We therefore must be aware that the adaptation of environmental bacteria to certain protozoa species may generate new pathogens. This speculation is supported by the observation that the respiratory pathogens *Chlamydia pneumoniae* and *Simkania negevensis* multiply within *Acanthamoeba* under laboratory conditions (Essig *et al*. 1997, Kahane *et al*. 2001). Since *Waddlia* was isolated from an aborted bovine fetus but also multiplies within protozoa it is conceivable that this microbe also falls into the group of environmentally preadapted pathogens.

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REFERENCES

Amann R. Springer N., Schönhuber W., Ludwig W., Schmid E. N., Müller K.-D., Michel R. (1997) obligate intracellular bacterial parasites of acanthamoebae related to *Chlamydia* spp. *Appl. Environ Microbiol*. **63:** 115-121

Corsaro D., Venditti D., Valassina M. (2002) New chlamydial lineages from freshwater samples. *Microbiology* **148:** 343-344

De Jonckheere J. F. (1977) Use of an axenic medium for differentiation between pathogenic an non-pathogenic *Naegleria fowleri* isolates. *Appl. Environ. Microbiol*. **33:** 751-757

Dilbeck P. M., Evermann J. F., Crawford T .B., Ward A. C. S., Leathers C. W., Holland C. J., Mebus C. A., Logan L. L., Rurangirwa F. R., McGuire T.C. (1990) Isolation of a previously undescribed rickettsia from an aborted fetus. *J. Clin. Microbiol*. **28:** 814-816

Essig A., Heinemann M., Simnacher U., Marre R. (1997) Infection of *Acanthamoeba castellanii* by *Chlamydia pneumoniae*. *Appl. Environ. Microbiol*. **63:** 1396-1399

Everett K. D., Bush R. M., Andersen A. A. (1999) Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms. *Int. J. Syst. Bacteriol*. **49:** 415-440

Fritsche T. R., Gautom R. K., Seyedirashti S., Bergeron D. L., Lindquist T. D. (1993). Occurrence of bacteria endosymbionts in Acanthamoeba spp. isolated from corneal and environmental specimens and contact lenses. *J. Clin. Microbiol*. **31:** 1122-1126

Fritsche T. R., Sobek D., Gautom R. K. (1998) Enhancement of the *in vitro* cytopathogenicity by *Acanthamoeba* spp. following acquisition of bacterial endosymbionts. *FEMS Microbiol. Lett*. **166:** 231-236

Fritsche T. R., Horn M., Wagner M., Herwig R. P., Schleifer K.-H., Gautom R. K. (2000) Phylogenetic diversity among geographically dispersed Chlamydiales endosymbionts recovered from clinical and environmental isolates of *Acanthamoeba* spp. *Appl. Environ. Microbiol*. **66:** 2613-2619

Giménez D. F. (1964). Staining rickettsiae in yolk sac cultures. *Stain Technol*. **39:** 135-140

Görtz H.-D., Michel R. (2003) Bacterial Symbionts of Protozoa in Aqueous Environments - Potential Pathogens? In: Emerging Pathogens, Oxford Biology

Henning K., Schares G., Granzow H., Polster U., Hartmann M., Hotzel H., Sachse K., Peters M., Rauser, M. (2002) *Neospora caninum* and *Waddlia chondrophila* strain 2032/99 in a septic stillborn calf. *Vet. Microbiol*. **85:** 285-292

Horn M., Wagner M., Müller K.-D., Schmid E. N., Fritsche T. R., Schleifer K.-H., Michel R. (2000) *Neochlamydia hartmannellae* gen. nov., sp. nov. (*Parachlamydiaceae*), an endoparasite of the amoeba *Hartmannella vermiformis*. *Microbiology* **146:** 1231-1239

Kahane S., Dvoskin B., Mathias M., Friedmann M. G. (2001) Infection of *Acanthamoeba polyphaga* with *Simkania negevensis* and *S. negevensis* survival within amoebal cysts. *Appl. Environ. Microbiol*. **67:** 4789-4795

Michel R., Hauröder-Philippczyk B., Müller K.-D., Weishaar I. (1994) *Acanthamoeba* from human nasal mucosa infected with an obligate intracellular parasite. *Europ. J. Protistol*. **30:** 104-110

Michel R., Henning K. Steinert M., Hauroeder B., Hoffman R., Zoeller L. (2001) Cocultivation of free-living amoebae and the first European isolate of *Waddlia chondrophila* (Chlamydiales). X International Congress of Protozoology-ICOP, Salzburg, Austria, July 15-19

Page F. C. (1988) A New Key to Freshwater and Soil Gymnamoebae. Freshwater Biological Association, Ambleside

Poppert S., Essig A., Marre R., Wagner M., Horn M. (2002) Detection and differentiation of *Chlamydiae* by fluorescence in situ hybridization. *Appl. Environ. Microbiol*. **68:** 4081-4089

Rurangirwa F. R., Dilbeck P. M., Crawford T. B., McGuire T. C., McElwain T. F. (1999) Analysis of the 16S rRNA gene of microorganism WSU 86-1044 from an aborted foetus reveals that it is a member of the order Chlamydiales: Proposal of Waddliaceae fam. nov., *Waddlia chondrophila* gen. nov., sp. nov. *Int. J. Syst. Bacteriol*. **49:** 577-581

Stephens R. S. (ed.). (1999) Chlamydia: Intracellular Biology, Pathogenesis, and Immunity. American Society for Microbiology, Washington, D. C.

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