Request for an Opinion

Phylogenetic analysis and description of *Eperythrozoon coccoides*, proposal to transfer to the genus *Mycoplasma* as *Mycoplasma coccoides* comb. nov. and Request for an Opinion

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*Eperythrozoon coccoides*, an epierythrocytic organism that causes a mild haemolytic anaemia in laboratory and wild mice, currently is thought to be a rickettsia. To determine the relationship of this agent to other haemotrophic bacterial parasites, the 16S rRNA gene of this organism has been sequenced and it is shown by phylogenetic analysis that this wall-less bacterium is not a rickettsia but actually is a mycoplasma. This mycoplasma shares properties with and is closely related to the other uncultivated mycoplasmas that comprise a recently identified group, the haemotrophic mycoplasmas (haemoplasmas). The haemoplasma group is composed of former *Eperythrozoon* and *Haemobartonella* species as well as newly identified haemotrophic mycoplasmas. Haemoplasmas parasitize the surface of erythrocytes of a wide variety of vertebrate animal hosts and are transmitted mainly by blood-feeding arthropod vectors. Because both primary infections and chronic latent infections caused by this bacterium have been observed in many laboratories and this bacterium has been the subject of much experimental work, considerable information exists about this haemotrophic mycoplasma that may be applicable to other haemoplasmas. It is proposed that *Eperythrozoon coccoides* be reclassified as *Mycoplasma coccoides* comb. nov. A Request for an Opinion is submitted to the Judicial Commission of the International Committee on Systematics of Prokaryotes regarding this reclassification.

*Eperythrozoon coccoides* is a blood parasite of laboratory and wild mice and was one of the first discovered members (Schilling, 1928; Dinger, 1928, 1929) of what has emerged as a large and ubiquitous group of haemotrophic bacteria that infect a broad range of mammalian hosts including, occasionally, humans (Weinman, 1935, 1944; Kreier et al., 1984, 1992; Neimark et al., 2001). *E. coccoides*, like most members of the group, is vector-transmitted and produces a primary acute infection which is followed by a persistent latent infection. Diagnosis has been made by detecting organisms on erythrocytes in Romanowsky-type or acridine orange-stained blood smears.

For decades the widespread occurrence of *E. coccoides* in laboratory mouse colonies, mouse tumour lines and protozoa research stocks was an unrecognized and serious problem because inapparent infections invalidated the results of numerous studies (Eliot, 1936; Gledhill, 1962; Riley et al., 1964; Baker et al., 1971).

*K. coccoides* was thought to be a rickettsia because of its obligate parasitism, small size and filterability, erythrocyte localization and arthropod vector transmission. *K. coccoides*, together with a number of similar haemotrophic bacteria, was classified in the order *Rickettsiales*, family *Anaplasmataceae* (Kreier & Ristic, 1984; Kreier et al., 1984, 1992). Recently, phylogenetic analysis of 16S rRNA gene sequences from several of these bacteria has demonstrated that these uncultivated wall-less bacteria are not rickettsia, but instead are mycoplasmas (Rikihisa et al., 1997; Neimark & Kocan, 1997; Neimark et al., 2001, 2002b). In addition, several new haemotrophic *Mycoplasma* species have been identified in wild and domesticated animals (Messick et al., 2002; Neimark et al., 2002b, 2004). Here we show that *E. coccoides*...
also is a Mycoplasma. Because numerous natural infections have been observed in a laboratory setting and this organism has been the subject of much research, considerable information exists. Consequently, this organism provides a general model to guide studies of haemotrophic mycoplasmas, particularly the pathogenic species that similarly produce chronic latent infections.

**PCR amplification, 16S rRNA gene sequencing, sequence analysis and phylogenetic tree construction**

Blood from mice infected with *E. coccoides* was collected with heparin and diluted 1:3 in Alsever’s/glycerol solution and cryopreserved at −70 °C. The blood was thawed and 0.2 ml inoculated by the intraperitoneal route into *Eperythrozoon*-free, random-bred, albino Swiss mice that had been inoculated 2 days earlier with *Plasmodium yoelii* strain NS1100. By 2 days after the *E. coccoides* inoculation, there was a heavy *E. coccoides* infection and blood samples were collected in acid/citrate/dextrose and approximately 50 μl applied to FTA filter papers (treated filter paper for collecting and storage of blood or other biological samples for subsequent DNA analysis; Fitzco), air-dried and stored at ambient temperature until processed. Three-millimetre-diameter circles were punched from the filters and each circle was transferred to a PCR tube, washed at room temperature three times with 500 μl of FTA Purification Reagent (Fitzco) for 10 min each, and then washed twice with 500 μl of TE (10 mM Tris, 1 mM disodium EDTA, pH 8-0) for 10 min each and air-dried. The *E. coccoides* 16S rRNA gene was amplified, and the gene sequence was determined with 16S rRNA gene primers as described previously (Neimark & Kocan, 1997; Neimark et al., 2002a). Comparison of the 16S rRNA gene sequence of *E. coccoides* to the GenBank database using BLASTN showed that the highest similarity scores matched to haemotrophic mycoplasmas.

Mycoplasma 16S rRNA gene sequences were obtained from GenBank. Alignments were made with the program CLUSTAL W, adjusted manually, and phylogenetic analysis of the sequence alignment was carried out with the phylogenetic program package PHYLIP 3.51c as described previously (Neimark & Kocan, 1997; Neimark et al., 2002a). The phylogenetic tree was constructed using the neighbour-joining method corrected for nucleotide substitutions by the Kimura two-parameter option with the transition/transversion ratio set at 2. The dataset was resampled 1000 times and the resulting bootstrap percentage values are indicated at the nodes of the tree shown in Fig. 1.

Phylogenetic analysis produced an evolutionary tree showing the relationship between *E. coccoides*, other haemotrophic mycoplasmas, representative species of the pneumoniae group and representatives of other mycoplasma groups (Fig. 1). The tree indicates that the *E. coccoides* 16S rRNA gene sequence clusters together with

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**Fig. 1.** Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of *E. coccoides* (*M. coccoides*) to other haemotrophic mycoplasmas and to some members of the pneumoniae group as well as to species representing other phylogenetic groups of mycoplasmas. Strain designations where reported and GenBank/EMBL/DDBJ accession numbers are included. *Acholeplasma laidlawii* was used as outgroup. Bootstrap confidence level percentage values obtained from 1000 resamplings of the dataset are shown at the nodes. Bar, distance equivalent to 1 substitution per 10 nucleotides.
all the other previously identified haemotropic mycoplasmas on a branch related to the pneumoniae group of the genus *Mycoplasma* (Neimark & Kocan, 1997; Johansson *et al*., 1999; Neimark *et al*., 2001).

The high bootstrap percentage values at most of the branch points in the phylogenetic tree (Fig. 1) indicate the probability that this is a robust tree. However, the few low bootstrap values (values below 50) at some branch points suggest these branches may be due to chance. The phylogenetic tree shows that *E. coccoides* (*Mycoplasma coccoides* comb. nov.) falls in the haemofelis cluster of the haemoplasmas where it is related to, but is clearly distinct from, *Mycoplasma haemomuris* (85 % similarity), a more pathogenic eperythrocitic mycoplasma found in laboratory rats and mice and in wild mice. Serum from mice infected with *E. coccoides* has been reported to react with *Haemobartonella muris* (*M. haemomuris*) and *Anaplasma marginale* antigens (Wigand, 1958). It is interesting to note that these two rodent mycoplasmas cluster phylogenetically with *Mycoplasma haemofelis*, a parasite of cats, which are rodent predators.

*E. coccoides* was first identified in laboratory mice (Schilling, 1928; Dinger, 1928) but wild mice probably are natural hosts (Bruymoghe & Vassiliadis, 1929a, b), and rats and rabbits have been infected experimentally (Bruymoghe & Vassiliadis, 1929c; Eliot & Ford, 1930). Natural infections with *E. coccoides* are transmitted by adults and nymphs of the lice *Polyplax spinulosa* (Eliot, 1936) and *Polyplax serrata*, which can transmit an infection after fasting for as long as 24 h (Weinman, 1944; Berkenkamp & Wescott, 1988); there is no evidence of *E. coccoides* replication in lice and transmission appears to be mechanical. Fleas, the rat louse and the mites *Myobia musculi*, *Mycoptes musculinus* or *Radfordia affinis* are not vectors (Eliot, 1936; Berkenkamp & Wescott, 1988). Experimental infections are produced usually by intravenous or intraperitoneal inoculation of infected blood and by oral administration of citrated blood but attempts at transmission by smearing mucous membranes (eyes, nostrils and urogenital surfaces) with citrated blood were not successful (Thurston, 1955).

Infections produce an intense parasitaemia that peaks on day 2 to 5 (Thurston, 1955; Gledhill *et al*., 1965a; Peters, 1965; Ott *et al*., 1967; Glasgow *et al*., 1971, 1974) when the number of organisms can exceed $10^9$ (ml blood$^{-1}$) (Stansly & Neilson, 1965). Subsequently, the parasitaemia declines rapidly so by day 6 or 7 the number of organisms in the peripheral circulation is very low and organisms may go undetected in blood smears unless they are specifically searched for. The acute phase displays a haemolytic anaemia with the haematocrit falling from 50–55 % to 20–30 % but by day 8 haematocrit levels return to the normal range (Glasgow *et al*., 1971). A marked splenomegaly is a characteristic of the infection and there is a general lymphadenopathy (Marmorston, 1935; Ansari *et al*., 1963; Riley, 1964). There are no obvious clinical symptoms and mortality is rare.

A latent infection ensues which persists for months and possibly for the life of the animal but there is no recognizable disease. Splenomegaly also persists in carrier mice presumably as long as they are infected (Baker *et al*., 1971) and low numbers of organisms may appear sporadically for brief periods in the peripheral blood of chronic carriers (Thurston, 1955). The existence of a latent infection is usually revealed by splenectomy which results in the reappearance of large numbers of organisms in the peripheral blood within 48 h; lower numbers of organisms then persist in the peripheral blood for several months (Schilling, 1928; Eliot & Ford, 1930; McCluskie & Niven, 1934; Marmorston, 1935; Thurston, 1954, 1955).

A notable finding is that *E. coccoides* infection raised phagocytic activity far above normal (Gledhill *et al*., 1965a). Also, there is a marked suppression of the interferon response (by 80 to 95 %) beginning 48 to 72 h after infection and continuing for 3 to 4 weeks (Glasgow *et al*., 1971, 1974). The capacity to produce interferon returned to normal by 6 weeks (Glasgow *et al*., 1971, 1974), so the interferon suppression lasted from about the end of the acute phase through the early latent phase. This response differs from a transient interferon response caused by *E. coccoides* infection (Glasgow *et al*., 1971; Suntharasamai & Rytel, 1973) that was probably due to release of pre-formed interferon (Glasgow *et al*., 1971).

During the acute phase infection there is a clear temporal relationship between the rapid disappearance of organisms from the blood and the rise of antibody (Hyde *et al*., 1972; Glasgow *et al*., 1974). In spite of this relationship, it is a question whether neutralizing antibody has a major part in maintaining latency since splenectomy results in rapid reappearance of organisms in the peripheral circulation. Although host defences contain the infection, there is a standoff between the host and the parasite: the host suppresses the parasitaemia and is resistant to superinfection (Thurston, 1955; Glasgow *et al*., 1974), yet the host is not able to eliminate the organisms and the infection persists.

Undetected *E. coccoides* infections in laboratory mouse colonies, mouse tumour lines and related materials have resulted in numerous cases of anomalous experimental findings and led to recognition that *E. coccoides* can cause complex pathophysiological changes that affect the host response to infection and that concurrent infections with *E. coccoides* and another infectious agent can markedly
alter the course of infection. Examples include conversion of the benign hepatitis produced by mouse hepatitis virus MHV1 to a fatal infection by a concurrent infection with *E. coccoides* (Niven et al., 1952; Gledhill, 1956, 1962; Seamer, 1967); the increase in mortality from lymphocytic choriomeningitis virus infection in mice concurrently infected with *E. coccoides* (Seamer et al., 1961; Seamer & Gledhill, 1965; Niven, 1971); the 'spleen weight increase factor' and other effects encountered during tumour transmission studies in mice (Nelson, 1956; Stansly, 1965; Stansly & Neilson, 1965, 1966a, b); the discovery of the lactate dehydrogenase-elevating virus (Arison et al., 1963; Riley, 1964, Riley et al., 1964; Fitzmaurice et al., 1972); and the discovery that *E. coccoides* infection increased the virulence of *Salmonella typhimurium* because normally harmless amounts of endotoxin produced high mortality in *E. coccoides*-infected mice (Gledhill & Niven, 1957; Niven, 1971).

In contrast, Peters (1965, 1987) showed that concurrent infections with *E. coccoides* and *Plasmodium berghei* resulted in a plasmodistatic effect that permitted longer survival of mice. Analogous effects were found with *E. coccoides* and additional plasmodia (Ott & Stauber, 1967; Ott et al., 1967; Voller & Bidwell, 1968; Cox, 1975; Suntharasamai & Marsden, 1969, 1970), with *Babesia rodhaini* (Peters, 1965) and *Trypanosoma brucei* (Bidwell & Voller, 1967). Also, *E. coccoides* protected mice from the lethal effects of Simliki Forest virus (Voller & Bidwell, 1968).

Finerty et al. (1973) showed that concurrent *E. coccoides* infection reduced the antibody response of mice to *P. berghei* and suggested antigenic competition could be responsible for this effect. Subsequently, Ljungström et al. (1974) showed that mice with an *E. coccoides* acute infection or anaplastic chronic infection when immunized with sheep red blood cells (SRBCs) had enhanced IgM plaque-forming capacity (acutely infected mice also had enhanced IgG capacity); however, they noted the possibility could not be excluded that the increase in plaque capacity was due to cross-reactivity between SRBCs and an *E. coccoides* antigen.

The question has been raised as to whether some animal mycoplasmas, such as *Mycoplasma pneumoniae*, which can attach to erythrocytes in vitro and are known to enter the blood circulation can also attach to erythrocytes in vivo (Neimark et al., 2001). Mucous membranes are the primary tissue habitat for animal mycoplasmas but whether haemotrophic mycoplasmas can also colonize mucous membranes is unknown.

*E. coccoides* has not been cultivated on artificial media. Derrick et al. (1954) found *E. coccoides* survived up to 9 days in embryonated chicken eggs but attempts at passage were unsuccessful; serial passages in embryonated chicken eggs have been reported (Seamer, 1959) but not confirmed. The standard procedure for determining whether a mouse colony was infected was to splenectomize a dozen 3- to 4-month-old mice and examine their blood daily (Gledhill et al., 1965b). An indirect fluorescent-antibody test has been shown to be useful (Baker et al., 1971) and scanning electron microscopy has been suggested to be useful (Augsten, 1982) but a PCR assay would be preferred, especially for studies of latency.

*E. coccoides* is readily killed by antiseptics and various chemical agents (Thurston, 1955; Stansly & Neilson, 1966b). The organism, like all mycoplasmas, is completely resistant to penicillin and other antimicrobial agents that target the cell wall. Tetracycline treatment is effective in reducing the number of organisms in the peripheral circulation but, based on experience with other haemoplasmas, this antibiotic probably does not eradicate the organism from infected animals. Organic arsenicals have been shown to be effective for treating *E. coccoides* (Bruymoghe & Vassiliadis, 1929b) and Neosarsaphenamine (300 μg per 20 g mouse) eradicated active and latent infections in mice (Thurston, 1953); these drugs have proved generally useful for haemoplasma infections (Michel et al., 2000).

It is proposed that *E. coccoides* be transferred to the genus *Mycoplasma* as *Mycoplasma coccoides* comb. nov., with the specific epithet ‘coccoides’ retained for continuity. Because this proposed transfer raises the taxonomic problems described below, we submit a Request for an Opinion of the Judicial Commission of the International Committee on Systematics of Prokaryotes and ask (i) the current use of the genus name *Mycoplasma* be maintained and (ii) *Mycoplasma coccoides* be considered a legitimate name.

(i) *Eperythrozoon* (Schilling, 1928) predates *Mycoplasma* (Nowak, 1929), consequently under Rules 15, 17, 44 and 51b the name of the united genus should be *Eperythrozoon*. We request an exception to the rules that would require renaming *Mycoplasma* species to *Eperythrozoon*, since changing the names of more than 100 *Mycoplasma* species to *Eperythrozoon* (as well as changing the order and family names from *Mycoplasmatales* and *Mycoplasmataceae*) would produce enormous confusion. In support of this request we cite Appendix 8 which states ‘In those cases where strict adherence to the rules of nomenclature would produce confusion or would not result in nomenclatural stability, exceptions to the rules may be requested of the Judicial Commission of the ICSB.’ With respect to confusion, it should be noted that over the past few years the transfer of the five other *Eperythrozoon* and *Haemobartonella* species to the genus *Mycoplasma* and the naming of several newly discovered haemotrophic bacteria as *Mycoplasma* has been well accepted and appears to have caused no confusion among veterinarians, the community most affected.

In addition, transferring all *Mycoplasma* species to *Eperythrozoon* would produce perilous names (Rule 56a). Problems could arise with mycoplasmas that cause serious human disease and because some animal pathogenic mycoplasmas are subject to quarantine regulations, changing their names would require name changes in quarantine regulations and documents around the world.
When the genus name *Eperythrozoon* was created with *Eperythrozoon coccoides* in 1928, there were no molecular identification or electron microscopic methods so it is not surprising that investigators did not recognize the fundamental characters shared by this bacterium and the bovine pleuropneumonia organism, then already known for more than 30 years, and the mycoplasma causing contagious agalactiae identified 3 years earlier. The incorrect identification of this and other haemotropic mycoplasmas as rickettsia (the genera *Eperythrozoon* and *Haemobartonella*) has long caused misunderstanding, so it is especially important that their genus name clearly convey their phylogenetic affiliation within the family *Mycoplasmataceae*.

(ii) Although *E. coccoides* clearly is a *Mycoplasma*, according to the Rules of the Bacteriological Code, the new combination *Mycoplasma coccoides* is illegitimate because *E. coccoides* is the type species of the genus *Eperythrozoon*. Ideally, this particular problem could be ameliorated by including in the proposed transfer the last remaining named species in the genus, *Eperythrozoon parvum* (all the other recognized species in the genus have been shown to be *Mycoplasma* species). However, this uncultured organism is not available and there is no assurance it can be found in a reasonable amount of time since one of us (H.N.) has been searching for a specimen for some years without success (requests to veterinary colleagues in North America and Europe to take a specimen if they encounter an infected pig have not resulted in any specimens). Except for three early papers on *E. parvum* 40 to 50 years ago, there has been only one other paper on this organism, nearly 25 years ago, and the description in Bergey’s Manual is quite limited. Since all the other species of *Haemobartonella* and *Eperythrozoon* in Bergey’s have now been shown to be *Mycoplasma* species, it seems likely *E. parvum* would prove to be a mycoplasma but there is no direct evidence and it cannot be determined when a specimen of this bacterium cannot be found and studied.

[After this manuscript was originally submitted we were advised to add a Request for an Opinion concerning these problems. As the revised manuscript was being submitted, Uilenberg et al. (2004) proposed on the basis of priority that all *Mycoplasma* species should be renamed *Eperythrozoon* but at the same time proposed waving the requirement that the order (*Mycoplasmatales*) and the family (*Mycoplasmataceae*) names be changed.]

**Description of Mycoplasma coccoides comb. nov.**

*Mycoplasma coccoides* (coc.coi’d.es. Gr. n. coccus a berry; N.L. n. coccus a coccus; Gr. n. eidis shape; N.L. adj. coccoides coccus-shaped).


Organisms in peripheral blood are coccoid and about 350 nm in diameter. Their association with the erythrocyte does not produce a noticeable indentation in the erythrocyte surface (Tanaka et al., 1965). Many organisms also are free in the plasma, especially early in the infection. Ring-shaped cells commonly observed in stained smears are an artefact of the staining process which suggests these organisms are easily deformed from their cocoid form. The cells are filterable through at least a 360 nm mean pore size colloidon filter (Niven et al., 1952). An epierythrocytic parasite of laboratory and wild mice, albino rats, hamsters and rabbits. Organic arsenicals (Neoarsphenamine; Oxophenarsine) are effective therapeutic agents that can eradicate a latent infection (Thurston, 1953; Thompson & Bayles, 1966; Powers et al., 1969); tetracyclines only suppress infection. The organism can influence the course of viral or protozoan infection in experimentally co-infected mice. Reported from Europe, North and South America and Africa. The louse *Polyptax spinulosa* is a vector. The organism has not been cultured on artificial media. Organisms in citrated blood remain infective for several hours at 16 °C and approximately 11 days at 3 °C (Thurston, 1955) and are maintained by animal passage, and storage at −70 °C. DNA G+C content (mol%) not determined. Genome size not determined.

Because this bacterium is uncultivated, no type strain is established. However, it is suggested that, where possible, chromosomal DNA from this and other uncultivated bacteria be deposited in a type culture collection to provide a type strain reference.

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**References**


