

# Proposal to reclassify *Ehrlichia muris* as *Ehrlichia muris* subsp. *muris* subsp. nov. and description of *Ehrlichia muris* subsp. *eaucloirensis* subsp. nov., a newly recognized tick-borne pathogen of humans

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## Abstract

We have previously described a novel taxon of the genus *Ehrlichia* (type strain Wisconsin<sup>T</sup>), closely related to *Ehrlichia muris*, that causes human ehrlichiosis among patients with exposures to ticks in the upper midwestern USA. DNA from this bacterium was also detected in *Ixodes scapularis* and *Peromyscus leucopus* collected in Minnesota and Wisconsin. To determine the relationship between the *E. muris*-like agent (EMLA) and other species of the genus *Ehrlichia* phenotypic, genotypic and epidemiologic comparisons were undertaken, including sequence analysis of eight gene loci (3906 nucleotides) for 39 EMLA DNA samples and the type strain of *E. muris* AS145<sup>T</sup>. Three loci were also sequenced from DNA of nine strains of *E. muris* from mouse spleens from Japan. All sequences from *E. muris* were distinct from homologous EMLA sequences, but differences between them were less than those observed among other species of the genus *Ehrlichia*. Phenotypic comparison of EMLA and *E. muris* revealed similar culture and electron microscopic characteristics, but important differences were noted in their geographic distribution, ecological associations and behavior in mouse models of infection. Based on these comparisons, we propose that type strain Wisconsin<sup>T</sup> represents a novel subspecies, *Ehrlichia muris* subsp. *eaucloirensis*, subsp. nov. This strain is available through the Centers for Disease Control and Prevention Rickettsial Isolate Reference Collection (CRIRC EMU002<sup>T</sup>) and through the Collection de Souches de l'Unité des Rickettsies (CSURP2883<sup>T</sup>). The subspecies *Ehrlichia muris* subsp. *muris* subsp. nov. is automatically created and the type strain AS145<sup>T</sup> is also available through the same collections (CRIRC EMU001<sup>T</sup>, CSUR E2<sup>T</sup>). Included is an emended description of *E. muris*.

The genus *Ehrlichia* includes multiple species of Gram-negative, non-motile, coccoid to ellipsoidal, obligate intracellular tick-borne bacteria that reside within cytoplasmic vacuoles of haematopoietic or endothelial cells in mammals [1]. Ehrlichiae are not cultivable in cell-free media but most can be isolated in one or more haematopoietic, endothelial or tick-derived cell lines [1]. There are currently five species with validly published names in this genus including the type species

*Ehrlichia canis* [2, 3], *Ehrlichia chaffeensis* [4], *Ehrlichia ewingii* (Anderson et al., 1992), *Ehrlichia muris* [5] and *Ehrlichia ruminantium*, [1, 6, 7].

We have reported previously the detection of a novel taxon of the genus *Ehrlichia*, type strain Wisconsin<sup>T</sup>, in whole blood specimens from four febrile patients with histories of tick exposures in the upper midwestern United States using culture, serology and PCR [8]. DNA from this bacterium was

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**Abbreviations:** CDC, Centers for Disease Control and Prevention; EMLA, *Ehrlichia muris*-like agent; IFA, immunofluorescence assay; EM, electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for *Ehrlichia muris* subsp. *eaucloirensis* are HM543745 (*rrs*), HQ660496 (*gltA*), KF523727 (*p13*), HQ660493 (*groEL*), KU672594 (*dsb*), KU672595 (*fbpA*), KU672596 (*nadA*), KU672597 (*p28-14*) and KU672598 (*p28-19*).

One supplementary table is available with the online Supplementary Material.

detected in *Ixodes scapularis* (black-legged ticks) [8–10] and blood of *Peromyscus leucopus* collected in Minnesota and Wisconsin [11].

Wisconsin<sup>T</sup> was originally described as representing a novel species of the genus *Ehrlichia* based on analyses of the partial *groEL* and 16S rRNA gene sequences [8]. These genes exhibited approximately 98 % sequence identity to homologous regions of the genome of *Ehrlichia muris* [8] thereby leading to the commonly applied moniker ‘*E. muris*-like agent (EMLA)’ that has been used to describe this bacterium since its initial characterization. While this member of the genus *Ehrlichia* has only been detected in the upper midwestern USA, *E. muris* is considered to be an Old World pathogen, found in Eastern Europe and Japan [12–17]. Recently, genotypic, phenotypic and epidemiological comparisons of EMLA Wisconsin<sup>T</sup>, *E. muris* and other species of the genus *Ehrlichia* prompted us to re-examine our prior conclusion that Wisconsin<sup>T</sup> represented a novel species of the genus *Ehrlichia*. This communication describes the results of this comparison and proposes that Wisconsin<sup>T</sup> represents a novel subspecies, *E. muris* subsp. *eaucloirensis* subsp. nov., and that the organism represented by the type strain AS145<sup>T</sup> be renamed *E. muris* subsp. *muris*, subsp. nov.

To better understand the degree of genetic variability found between and within species of the genus *Ehrlichia*, we analyzed sequences of eight gene loci (*gltA*, *groEL*, *nadA*, *dsb*, *fbpA*, *p13*, *p28-14*, *p28-19*) comprising 3906 nucleotides from EMLA-positive DNA samples, including Wisconsin<sup>T</sup>, *E. muris* AS145<sup>T</sup> (ATCC, VR-1411) and other species of the genus *Ehrlichia* (Table 1 and S1, available in the online Supplementary Material), [9, 18–21]. EMLA-positive DNA extracts were obtained from 21 humans, 17 *I. scapularis* and 1 *P. leucopus*. Three loci (16S, *groEL* and *p28-14*; 1559 nt) were also sequenced for nine *E. muris*-positive DNA extracts obtained from mouse spleens at Osaka Prefecture University, Japan and compared with the *E. muris* type strain, AS145<sup>T</sup>. PCR products were sequenced in both directions, and sequencing reads were assembled using Sequencher 5.1 (Gene Codes). Sequences were aligned using

MEGA 5.1 ([www.megasoftware.net/](http://www.megasoftware.net/)) and percentage identity was determined using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). An estimation of the evolutionary history of *E. muris* subsp. *eaucloirensis* compared with other established ehrlichial species was inferred using MEGA 5.1 ([www.megasoftware.net/](http://www.megasoftware.net/)) by trimming the PCR primer sequences and concatenating the sequences for *gltA*, *groEL*, *nadA*, *dsb*, *fbpA*, *p28-14* and *p28-19*. Indels were removed using the simple indel coding method [22]. EMLA Wisconsin<sup>T</sup> was found to be closely related to *E. muris* AS145<sup>T</sup> (Fig. 1), with a shorter branch length than found between existing ehrlichial species. Diversity within EMLA samples was seen for only one locus (*p28-14*), in which one EMLA-positive sample (UW-M7) was indistinguishable from *E. muris* (GenBank accession DQ335244). Similarly, of the three *E. muris* loci, no diversity was found among the nine samples tested and all were identical to *E. muris* AS145<sup>T</sup>. However, all *E. muris* sequences were distinct from the homologous sequences of EMLA. The level of genetic similarity between EMLA and *E. muris* AS145<sup>T</sup> at these eight loci was compared with those observed among four distinct species of the genus *Ehrlichia* at the same loci (Table 1). From these data, we determined that the genetic differences between EMLA and strains of *E. muris* at these loci were less than those observed among multiple recognized species of the genus *Ehrlichia*.

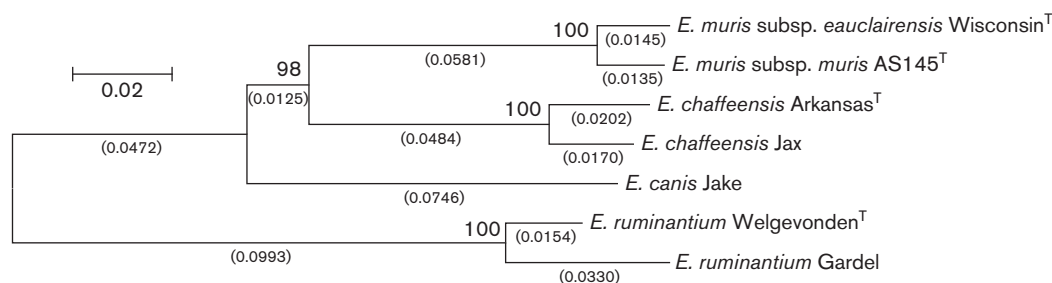
A phenotypic comparison of EMLA Wisconsin<sup>T</sup> and *E. muris* AS145<sup>T</sup> was also performed using cell culture, electron microscopy (EM) and animal inoculation. EMLA Wisconsin<sup>T</sup> and *E. muris* AS145<sup>T</sup> [5] were propagated in DH82 canine macrophage-like cells (American Type Culture Collection number CRL-10389) at 37 °C in a 5 % CO<sub>2</sub> atmosphere. The cells were fed with minimal essential medium (MEM) (Gibco) supplemented with 0.1 mM MEM non-essential amino acids (Gibco), 10 mM HEPES buffer (Gibco), 2 mM L-glutamine (Gibco), 10 mM sodium pyruvate (Gibco) and 10 % heat-inactivated fetal bovine serum (Atlanta Biologicals). When grown in DH82 canine macrophage cells and stained with the Jorvet Dip Quick stain

**Table 1.** Comparison of strain Wisconsin<sup>T</sup> to strains of other species of the genus *Ehrlichia*

Amplicon lengths are shown in the online Supplementary Material. The following GenBank files were used in this analysis (*E. chaffeensis* Arkansas genome NC\_007799, *E. canis* Jake genome NC\_007354, *E. ewingii* *gltA* DQ365879, *groEL* AF195273, *dsb* KM458249, *p28-14* EF116932 and *p28-19* EF116932 and *E. ruminantium* Welgevonden<sup>T</sup> genome NC\_005295.2).

	Percentage identity of strain Wisconsin <sup>T</sup> to other species of the genus <i>Ehrlichia</i>							
	<i>gltA</i>	<i>groEL</i>	<i>nadA</i>	<i>dsb</i>	<i>fbpA</i>	<i>p13</i>	<i>p28-14</i>	<i>p28-19</i>
<i>E. muris</i> AS145 <sup>T</sup>	98	98	98	96	96	90	95	95
<i>E. chaffeensis</i> Arkansas <sup>T</sup>	88	94	89	87	91	*	83	80
<i>E. canis</i> Jake	87	93	89	82	88	*	82	77
<i>E. ewingii</i>	83	91	*	78	*	*	76	81
<i>E. ruminantium</i> Welgevonden <sup>T</sup>	79	88	84	79	82	*	78	77

\*Indicates genetic data unavailable in GenBank.



**Fig. 1.** The evolutionary history was inferred using the neighbor-joining method [34]. The optimal tree with the sum of branch lengths=0.45356965 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches [35]. The tree is drawn to scale with branch lengths, shown under the branches (in parentheses), in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [36] and are in the units of the number of base substitutions per site. The analysis involved seven nucleotide sequences and all positions with less than 95 % site coverage were eliminated. There were a total of 3781 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [37]. The following GenBank files were used in this analysis (*E. chaffeensis* Arkansas<sup>T</sup> genome NC\_007799, *E. chaffeensis* Jax genome NZ\_CP007475.1, *E. canis* Jake genome NC\_007354, *E. ruminantium* Welgevonden<sup>T</sup> genome NC\_005295.2, and *E. ruminantium* Gardel genome NC\_006831.1).

(Jorgensen Laboratories), EMLA appears as small clusters of bacteria, known as morulae, within vacuoles in the host cell cytoplasm (Fig. 2a), and no growth differences were observed between EMLA Wisconsin<sup>T</sup> and *E. muris* AS145<sup>T</sup>.

To fix cells for transmission EM analysis, an infected DH82 monolayer was washed in 0.1 M phosphate buffer, pH 7.3 and fixed in buffered 2.5 % glutaraldehyde for 5 min at 4 °C. Cells were detached from the monolayer by using a cell scraper, and then centrifuged at 2500 r.p.m. for 5 min at 4 °C and allowed to fix for 10 min. The glutaraldehyde was removed and fresh phosphate buffer was layered onto the pellet, which was stored at 4 °C. The cells were post-fixed in 1 % buffered osmium tetroxide, stained in 4 % uranyl acetate, dehydrated through a graded series of alcohols and acetone and embedded in a mixture of Epon-substitute and Araldite [23]. Thin sections were stained with 4 % uranyl acetate and Reynold's lead citrate. As described for other species of the genus *Ehrlichia*, intracellular EMLA bacteria exist as two forms, reticulate cells (RC) and dense-cored cells (DC), [24] (Fig. 2b). Both cell types appear slightly oblong, with dense-cored cells averaging 700×403 nm while the reticulate cells are a little larger, with an average cell size of 964×458 nm. The morulae averaged 1.42×1.27 μm and usually consisted of a homogeneous population of either cell type. Morulae contained a fibrillary matrix of varying density (Fig. 2d) and host cell mitochondria were often found in proximity to morulae (Fig. 2b). Reticulate cells occasionally demonstrated long projections of the bacterial cell membrane that invested other ehrlichial cells in morulae (Fig. 2c). Similar findings were observed for *E. muris* AS145<sup>T</sup> (data not shown) and have been previously described when AS145<sup>T</sup> was grown in other cell lines [24].

Despite similarities in culture and EM characteristics, EMLA Wisconsin<sup>T</sup> and *E. muris* AS145<sup>T</sup> demonstrated pathogenic differences in mouse models of infection. *E. muris* AS145<sup>T</sup> causes a sub-lethal infection in C57BL/6 mice [25–27].

EMLA Wisconsin<sup>T</sup> can lead to either a lethal or persistent infection in the same mouse strain, depending on the route of infection [28, 29]. Additionally, EMLA Wisconsin<sup>T</sup> that was transmitted by ticks has also been shown to cause mouse mortality [29].

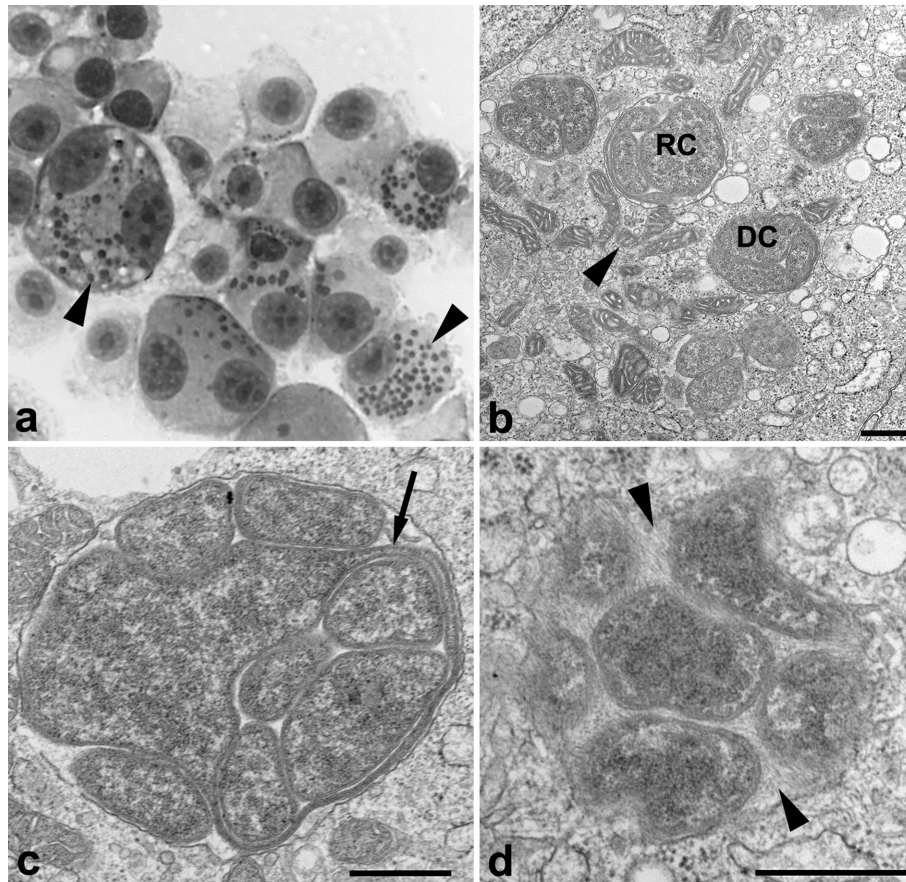
Finally, ecological analysis of EMLA Wisconsin<sup>T</sup> and *E. muris* AS145<sup>T</sup> reveals differences between the geographic distributions and host associations of these organisms. *E. muris* has been found in Japan [17], Russia [14], Slovakia [12] and Korea [16], while EMLA strains, including Wisconsin<sup>T</sup>, have thus far been found only in Minnesota and Wisconsin in the Western Hemisphere [8–10, 30]. *Ixodes persulcatus* and *Haemaphysalis flava* ticks serve as vectors of *E. muris* [12, 31], whereas EMLA Wisconsin<sup>T</sup> is transmitted by *I. scapularis* [28, 29, 32]. It is also apparent that EMLA causes human disease, while it is not known whether *E. muris* AS145<sup>T</sup> is a human pathogen, despite serological evidence of human exposure [31].

We conclude that EMLA Wisconsin<sup>T</sup> represents a novel subspecies of *E. muris* and propose naming it *E. muris* subsp. *eauclairensis* based on the geographic origin of the original isolate.

## DESCRIPTION OF *EHRLICHIA MURIS* SUBSP. *MURIS* SUBSP. NOV.

*Ehrlichia muris* subsp. *muris* (mu'ris. L. gen. n. *muris*, of a mouse; the subspecies was first isolated from a mouse).

*Ehrlichia muris* subsp. *muris* [5] has been found in *Ixodes persulcatus* and *Haemaphysalis flava* hard-bodied ticks, wild mice and sika deer (*Cervus nippon yesoensis*) in regions of Eastern Europe and Japan [13, 15, 20, 25, 31, 33]. There is also serological evidence of human, boar, dog, deer, bear and monkey infections in Japan [31], although it is difficult to determine if these antibodies



**Fig. 2.** EMLA in cultured cells. (a) Dip Quick stain of EMLA infecting DH82 canine macrophage cells ( $\times 100$ ) showing bacterial morulae (arrow heads). (b–d) Electron microscopic analysis of EMLA infecting DH82 cells, scale bars, 500 nm. (b) EMLA exhibits two cell types, dense-cored cells (DC) and reticulate cells (RC). Host cell mitochondria (arrowhead) are also found in close association with the morulae. (c) Long projection of RC ehrlichial cell membrane (arrow) that completely surrounds other RC. (d) A fibrillary matrix (arrow heads) of varying densities is often observed in EMLA morulae.

result from *E. muris* or other ehrlichial agents described in that region. The natural history of *E. muris* subsp. *muris* is incompletely characterized but probably involves small rodent hosts; wild caught specimens of infected *Eothenomys kageus* [26], *Apodemus flavicollis* [12], and *Apodemus speciosus*, and *Apodemus argenteus* [31] have been identified. In laboratory settings *E. muris* subsp. *muris* causes sub-lethal infections in BALB/c, DBA/2, C57BL/6, C3H, ICR, CBA and ddY [17] AKR [25], and BALB/c mice [26]. Protection against infection in mice appears to be mediated through a combination of CD4 and CD8 T lymphocytes, antibodies, tumor necrosis factor and interferon gamma, with lethal infection observed in CD4 and CD8 lymphocyte-depleted mice [25]. Infection is associated with a short-lived clinical illness and persists for the life of the mouse [25, 26]. The target cell(s) in naturally infected vertebrate hosts is unknown; however, ehrlichiae can be found in mononuclear cells of various organs and tissues and occasional hepatocytes in AKR and C57BL/6 mice experimentally infected with this organism [25].

The type strain, AS145<sup>T</sup>, is available through the Centers for Disease Control and Prevention Rickettsial Isolate Reference Collection (CRIRC EMU001<sup>T</sup>) and through the Collection de Souches de l'Unité des Rickettsies (CSUR E2<sup>T</sup>).

#### **DESCRIPTION OF *EHRlichia MURIS* SUBSP. *EAUCLAIRENSIS* SUBSP. NOV.**

*Ehrlichia muris* subsp. *eauclairensis* (eau.clair.en'sis. N.L. fem. adj. *eauclairensis*, from Eau Claire; the type strain was isolated from a patient from Eau Claire, Wisconsin, in 2009).

To date, all infected tick and vertebrate hosts have originated from Minnesota and Wisconsin. Human infection with *E. muris* subsp. *eauclairensis* causes an illness characterized by fever, headache, myalgias, lymphopenia and thrombocytopenia [8, 30]. *Ehrlichia muris* subsp. *eauclairensis* is serologically cross-reactive with *E. chaffeensis* as determined by IFA [8]. The target cell(s) in naturally

infected vertebrate hosts is unknown; however, ehrlichiae can be found in mononuclear and endothelial cells of various organs and tissues in mice experimentally infected with this organism [28]. *E. muris* subsp. *eauclairensis* is passaged transstadially in and transmitted by *I. scapularis* ticks [28, 29, 32] and the bacterium has been detected in or isolated from nymphal and adult stages [8–10, 32]. In the tick, *E. muris* subsp. *eauclairensis* infects multiple cell types, particularly epithelial cells of the salivary glands, tracheae and male accessory glands, as well as neuronal cells of the synganglion [32]. The natural history of *E. muris* subsp. *eauclairensis* is incompletely characterized but probably involves small rodent hosts; wild-caught specimens of infected *Peromyscus leucopus* have been identified [11]. In the laboratory setting *E. muris* subsp. *eauclairensis* has been shown to infect C57BL/6 mice, and is capable of causing a lethal infection in a dose-dependent manner. Although bacteremia only occurs for a short time, multiple organs are infected, including the lungs, liver and spleen [28, 29]. The bacteremia is sufficient for ehrlichial transmission to *I. scapularis* ticks through feeding in both C57BL/6 mice [28, 29] and Syrian hamsters (*Mesocricetus auratus*) [32] and tick transmission may lead to mouse mortality [29]. A draft genome assembly for strain EmCRT is available (NCBI NZ\_LANU01000000) consisting of 1.15 mb with 29.8% GC.

The type strain, Wisconsin<sup>T</sup>, is available through the Centers for Disease Control and Prevention Rickettsial Isolate Reference Collection (CRIRC EMU002<sup>T</sup>) and through the Collection de Souches de l'Unité des Rickettsies (CSURP2883<sup>T</sup>).

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#### Conflicts of interest

The authors declare that there are no conflicts of interest.

#### Ethical statement

Patient follow-up and DNA sequencing of clinical specimens was approved by the Mayo Clinic institutional review board. All animal experiments were performed in accordance with Nagoya Environmental Health Institute Institutional Animal Care and Use Committee guidelines and approved.

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