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Lack of serological evidence for *Mycoplasma fermentans* infection in army Gulf War veterans: a large scale case-control study

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SUMMARY

Mycoplasma fermentans is suspected in the development of 'Gulf War illness' in veterans of Operation Desert Storm. We conducted a matched case-control study for the prevalence of *M. fermentans*-specific antibodies before and after the operation, as well as seroconversion rates in veterans with and without complaints of 'Gulf War illness'. Cases consisted of Gulf War veterans, who complained of various illnesses and were enrolled in the second phase of the health evaluation by the Army Comprehensive Clinical Examination Program (CCEP). Controls were selected from Gulf War veterans who did not participate in the registry and did not request a health evaluation by the CCEP. Before operation deployment, 34 out of 718 of the cases (4·8%) and 116 out of 2233 of the controls (5·2%) tested positive for *M. fermentans*-specific antibodies. There was no difference in rates of seroconversion between cases and controls (1·1 *vs.* 1·2%) to *M. fermentans* during Operation Desert Storm. Thus, there is no serological evidence that suggests infection by *M. fermentans* is associated with development of 'Gulf War illness'.

INTRODUCTION

Mycoplasmas are a heterogeneous group of the smallest organisms capable of self-replication. They are known to cause a wide variety of diseases in animals [1]. Some mycoplasmas cause respiratory or urogenital diseases in humans [2]. However, other mycoplasmas chronically colonize human respiratory and urogenital tracts without apparent clinical significance [2]. Host and environmental factors may change an otherwise silent colonization into a prominent clinical outcome. Thus, mycoplasmas are suspected to play an important role in causing various chronic debilitating illnesses, particularly in patients with immune dysfunction. A major complication in assessing the role of mycoplasmas in disease processes is the difficulty in detecting fastidious mycoplasmas that fail to grow in current culture systems.

Several years ago, our laboratory first reported *Mycoplasma fermentans* (strain incognitus) in patients with AIDS [3–5]. We later reported, in non-HIV infected patients, a previously unrecognized fulminant disease apparently associated with systemic *M. fermentans* infection [6–9]. These highly unusual cases revealed a rapidly deteriorating illness with adult respiratory distress syndrome (ARDS) and/or multiple organ failure. These findings suggested that *M. fermentans* may be pathogenic in humans. However, although no other infectious agents could be iden-

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tified, it was not clear if these patients had another yet unknown underlying condition.

In recent years, many veterans who were deployed in Operation Desert Storm (ODS) presented to medical treatment facilities with a variety of medical complaints that have been difficult to define and to objectively document [10]. Most common complaints included fatigue, weight loss, myalgia, arthralgia, headaches, and short-term memory impairment. The cause(s), if any, is (are) not known for the broad spectrum of symptoms that have been collectively referred to by many as Gulf War Syndrome or Gulf War illness (GWI) [10, 11]. However, studies of GWI are seriously confronted by the fundamental problem of lacking a proper case definition for the 'illness', due to the wide spectrum of, often non-specific, health problems presented by the veterans [12]. Possible causes proposed by various investigators include exposure to pesticides, chemical warfare agents, vaccines, pyridostigmine bromide, petroleum products, oil-well fire smoke, stress, as well as infectious agents such as leishmania or biological warfare agents [13].

Some scientists and veterans have suggested that infection by mycoplasmas, in particular *Mycoplasma fermentans*, may cause many of the symptoms found in these veterans [14, 15]. It has been reported that more than 50% of veterans with GWI had *M. fermentans* (strain incognitus) in their blood as measured by a molecular diagnostic technique called Nuclear Gene Tracking (NGT) developed by Nicolson and Nicolson [15–17]. In one of the controlled studies reported by Nicolson and colleagues, 14/30 (47%) Gulf War veterans with GWI tested positive for *M. fermentans* compared with 0 of 21 (0%) healthy controls [15]. Furthermore, antibiotic treatment directed against mycoplasma (doxycycline) was reported to significantly improve symptoms in Gulf War veterans with GWI [14]. Because NGT is not a commonly used methodology to detect infectious agents in blood of patients, we felt it would be important to examine the *M. fermentans*-GWI hypothesis using a well-established and widely accepted laboratory technique for studying infectious diseases. Similar findings using a well-described approach would provide compelling evidence that *M. fermentans* is possibly an important cause of the ill-defined GWI.

Our laboratory has developed, using purified lipid-associated membrane proteins (LAMPs) as antigens, a highly sensitive and specific serological test that detects species-specific antibodies to mycoplasmas [18–22]. Our earlier studies of human antibody response to various species of mycoplasmas showed that most, although not all, produce detectable antibodies following mycoplasmal infections, even in immune compromised patients with AIDS [18, 19, 21, 22]. Although we do not expect every patient infected by the mycoplasma to produce detectable antibodies to *M. fermentans*, we believe most patients, similar to patients with AIDS, should mount a response. In order to better evaluate the *M. fermentans*–GWI hypothesis, we conducted a large scale case-control study to compare the prevalence of *M. fermentans*-specific antibodies between the Gulf War veterans with unexplained illness and a matched group of veterans who did not enroll in the registry for health evaluation. Furthermore, we analysed, using archived serum samples obtained on each individual before and after the deployment, the rates of seroconversion for this mycoplasma.

MATERIALS AND METHODS

Study population

The Department of Defense (DoD) has instituted a voluntary programme, the Comprehensive Clinical Examination Program (CCEP), to provide a systematic and uniform medical evaluation of Gulf War veterans' health [12]. Phase I of the CCEP is conducted at a local military medical treatment facility under the direction of a board-certified family practice or internal medicine physician. This evaluation consists of a complete physical examination, medical history, and laboratory tests that include a complete blood count, urinalysis, blood chemistry for electrolytes, glucose, creatinine, blood urea nitrogen, and liver enzymes. Further tests and consultations are done based upon the initial history, physical examination and laboratory results. If the diagnosis is still uncertain after Phase I evaluation, or if the patient is unsatisfied with the explanation from Phase I evaluation, patients are referred for Phase II evaluation. Most of the first 20000 patients (88%) enrolled in the programme were diagnosed in Phase I or were satisfied with their diagnosis. Thus, only 12% of patients were referred to Phase II of the evaluation [12]. Phase II is a more extensive evaluation conducted at 1 of 14 DoD regional medical centers. This evaluation requires a review of Phase I, consultations in infectious disease,

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psychology and psychiatry, and laboratory tests to include sedimentation rate, C-reactive protein, rheumatoid factor, fluorescent antinuclear antibodies, thyroid function, B12 and folate levels, creatine phosphokinase level, HIV antibody, hepatitis B serology and syphilis serology. Additional consultations and tests are obtained based on the initial Phase II evaluation.

Study design and case control definition

The study design was a matched case-control study. All soldiers who were on active duty from 1 August 1990 to 31 July 1991 and were deployed during ODS were eligible to be in the study. Cases consisted of Army Gulf War veterans who were enrolled in the Phase II study of the CCEP. Controls were randomly selected from Gulf War veterans who chose not to be enrolled on the registry for health evaluation. For each case, three controls were randomly selected from the pool of non-cases that met the matching criteria. Controls were matched to cases (3:1) by age (± 2 years); gender; the difference in the length of the interval between pre- and post-deployment serum specimens or 'specimen time interval' (± 31 days); and time in Gulf (± 31 days). Additional demographic information obtained on study subjects included race, marital status, rank, and status as reserve, active duty, or National Guard.

The possible serostatus categories based on the combination of pre- and post-deployment serum specimens were the following: negative [negative (0) on pre-deployment serum and negative (0) on the post-deployment specimen]; seroconversion [negative (0) on pre-deployment specimen and positive (1) on the post-deployment specimen]; seropositive [positive (1) on both specimens]; and seroreversions [positive (1) on the pre-deployment specimen and negative (0) on the post-deployment specimen]. This study was reviewed and approved by the human use committee and institutional review board at Walter Reed Army Medical Center and the Walter Reed Army Institute of Research.

Laboratory methods

Pre- and post-deployment serum was obtained from the DoD Serum Repository under the supervision of the US Army Center for Health Promotion and Preventive Medicine (USACHPPM). The selection of cases and controls and coding of serum specimens also was performed by USACHPPM. Laboratory personnel were blinded to the case-control status as well as the pre- and post-deployment status of the specimens.

Serum sample preparation, procedures for preparation of *M. fermentans* lipid-associated membrane proteins (LAMPs), ELISA and Western Blot (WB) techniques of studying antibodies to LAMPs have been described in detail previously (18–22). Briefly, 4 μg/ml *M. fermentans* LAMPs were coated on Nunc-Immuno F96 MaxiSorp plates with 100 μl in each well. After overcoating with 0·1% BSA, 100 μl human serum or plasma [diluted 1·250 in 10% normal goat serum, 2% BSA and 0·3% Nonidet P-40 (NP-40) in PBS] was added to each well. Subsequent steps included addition of 100 μl of 1/1000 biotin-labelled antibody of goat anti-human IgG-λ [Kirkegaard & Perry Laboratories (KPL) Inc.] and 1/20000 peroxidase-labelled streptavidin (KPL) that were prepared in 10% normal goat serum, 2% BSA, 0·1% NP-40, 1X PBS (Diluent I). The plates were developed by adding 100 μl of 2,2-β-azino-di-(3-ethyl-benzthiazoline-sulphonate)peroxidase substrate solution into each well. The plates were washed six times with PBS (pH 7·2) plus 0·05% NP-40 (Solution A) between each step. Coefficiency of variation (CV) of the ELISA test was obtained by measuring OD readings from four control specimens (one with positive antibodies and three with negative antibodies). Each of these positive and negative control samples were tested 82 times (observations) in the 4-month testing period. Intra-assay CV was 1·4–1·8% for the antibody-positive sample and 3·1–7·3% for the antibody-negative samples. Inter-assay CV was 5·3% for the positive sample and 15·9–20·2% for the negative samples. Determination of the cut-off value for positivity of *M. fermentans*-specific antibodies in our ELISA test was previously described in detail [20]. All samples were tested at least twice with duplication in the initial screening. Positive samples identified were then repeated in triplicate and tested in plates coated with BSA without LAMPs. All samples tested positive by ELISA were confirmed by WB [18].

In WB analysis, LAMPs (about 120 μg) from *M. fermentans* TX-114 extract were separated by SDS–polyacrylamide gel ($14 \times 12 \times 0{\cdot}75$ cm) electrophoresis and electroblotted on a BA-85 nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked with 10% fetal bovine serum and 1% BSA in

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PBS pH 7·2 and cut into 4 mm strips. Each strip was incubated with 2 ml of 1·250 human serum at 25 °C for 15 h with shaking. The strips were washed six times with Solution A, incubated at 25 °C with 1/1000 biotin-labelled antibody of goat anti-human IgG-γ for 3 h, incubated at 25 °C with 1/10000 peroxidase-labelled streptavidin in Diluent I for 90 min, and developed at 37 °C for 20 min with the 4-chloro-l-naphthol peroxidase substrate system (KPL).

Statistical analysis

Descriptive statistics including χ^2 and tests for mean differences were done using SAS statistical software [12]. Continuous variables included: 'time in the Gulf' and 'specimen time interval'. Age was divided into five categories (18–20, 21–25, 26–30, 31–35, $>$ 35). To assess the risk associated with enrollment in Phase II of the CCEP and pre-and post-deployment serostatus, odds ratios (OR) and 95% confidence intervals (95% CI), adjusted for several covariates, were obtained from logistic regression models using the SAS procedure CATMOD. In addition to the matching factors, other variables entered into the model included the following: race (white, black, other); marital status (single, married, other); rank (Junior Enlisted, Senior Enlisted, Officer, Warrant Officer); and component (regular Army, Army National Guard, Army Reserves). Initially, matched analyses were performed. Since results were similar when unmatched analyses were conducted, the results presented here are unmatched with control for various confounding factors.

RESULTS

There were 1364 Army Gulf War veterans in Phase II CCEP. Demographic information, deployment dates and serum specimens were available for 835 individuals. Of these individuals, controls were matched to 769 cases. Thus, the initial study population consisted of 769 cases and 2307 matched controls (1:3). Pre-deployment serum specimens were unavailable for 67 individuals and post-deployment serum specimens were not found for 64 individuals. Both specimens were unavailable for 6 of these individuals. After elimination of these study subjects, the analysis was based on 718 cases and 2233 controls.

As shown in Table 1, the distribution of the matching variables was similar for cases and controls. For both cases and controls, the mean age was 29 years, the average time in the Gulf was 167 days, and the average time interval between the pre- and post-deployment serum specimens was about 42 months (1268 and 1265 days, respectively). Approximately 16% of the study's population were female. Statistically significant differences between cases and controls were found for the unmatched variables race, marital status, rank and component. Compared to controls, cases were more likely to be non-white, married, and from senior enlisted. Cases also were slightly more likely to be regular Army.

Before deployment, 34 out of 718 of the cases (4·8%) and 116 out of 2233 of the controls (5·2%) tested positive for *M. fermentans*-specific antibodies (Table 2). Only 8 cases (1·1%) and 26 controls (1·2%) seroconverted to *Mycoplasma fermentans*. The unadjusted odds ratio for participation in phase II CCEP was 0·95 (95% CI = 0·43, 2·11) for Army Gulf War veterans who seroconverted compared to veterans who were seronegative on both serum specimens (Table 2). Of note, all of the seroconversions occurred in Gulf War veterans who were in the regular Army (Table 3).

In addition, Table 2 also presents the frequency of other serostatus categories as well as the unadjusted odds ratio comparing these serostatus categories to veterans who were negative on both pre- and post-deployment serum specimens. There were 17 cases (2·4%) and 54 controls (2·4%) who were seropositive on both the pre- and post-deployment serum specimens (OR = 0·97, 95% CI = 0·56, 1·69). Moreover, there were 17 cases (2·4%) and 62 controls (2·8%) who were seropositive on the pre-deployment serum specimen and negative on the post-deployment specimen (OR = 0·85, 95% CI = 0·49, 1·46).

In Table 4, Model 1 contains the matching variables as well as factors that were potential confounders. For this model, a statistically nonsignificant odds ratio for participation in phase II CCEP of 1·12 (95% CI = 0·32, 3·97) was found for Gulf War veterans who seroconverted compared to Gulf War veterans who were seronegative on both the pre- and post-deployment serum specimens. Similar results were found for Model 2 where only potential confounding factors were entered into the model (OR = 1·14, 95% CI = 0·32, 4·03). Since all of the seroconversions occurred only among regular Army Gulf War veterans, logistic regression analyses were performed restricted to this group. As noted in Table 5, the odds ratios were quite similar [Model 1: OR = 1·15

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Table 1. *Demographic characteristics of Army Gulf War veterans enrolled in Phase II CCEP (cases) and Army Gulf War veterans not enrolled in the registry (controls), Gulf War Veterans Mycoplasma Study*

	Cases ($n = 718$)		Controls ($n = 2233$)		*P*-value
	n	%	*n*	%	χ^2
Age*					
18–20	72	10·0	238	10·7	
21–25	156	21·7	569	25·5	
26–30	175	24·4	508	22·7	0·21
31–35	179	24·9	493	22·1	
> 35	136	18·9	425	19·0	
Gender*					
Male	604	84·1	1882	84·3	0·92
Female	114	15·9	351	15·7	
Race					
Black	230	32·0	494	22·1	
White	410	57·1	1552	69·5	0·001
Other	78	10·9	187	8·4	
Marital status					
Married	484	67·4	1345	60·2	
Single	184	25·6	771	34·5	0·001
Other	50	7·0	117	5·2	
Rank					
Jr Enlisted	259	36·1	808	36·2	
Sr Enlisted	384	53·5	980	43·9	0·001
Officer	61	8·5	345	15·5	
W-Officer	14	1·9	100	4·5	
Component					
Regular Army	676	94·2	2043	91·5	
Army National Guard	15	2·1	53	2·4	0·047
Reserves	27	3·8	137	6·1	
	Mean	S.D.	Mean	S.D.	*t* test
Specimen time interval (days)*	1268	499	1265	503	0·87
Time in Gulf (days)*	167	62	167	62	0·90

* Matching variables.

Table 2. *Unadjusted odds ratio for participation in Phase II CCEP associated with serostatus, Army Gulf War Veterans Mycoplasma Study*

Pre- and post-deployment serostatus	Cases ($n = 718$)		Controls ($n = 2233$)		Odds ratio	95% CI
	n	%	*n*	%		
Negative (0–0)	676	94·1	2091	93·6	1·00	
Seroconversion (0–1)	8	1·1	26	1·2	0·95	(0·43, 2·11)
Seropositive (1–1)	17	2·4	54	2·4	0·97	(0·56, 1·69)
Seroreversion (1–0)	17	2·4	62	2·8	0·85	(0·49, 1·46)

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Table 3. *The frequency of serostatus by component, Army Gulf War Veterans Mycoplasma Study*

Component	Negative (0–0)	Sero conversion (0–1)	Sero positive (1–1)	Sero reversion (1–0)	Total
Regular Army	2545	34	65	75	2719
Army National Guard	65	0	3	0	68
Army Reserves	157	0	3	4	164
Total	2767	34	71	79	2951

Table 4. *Logistic regression models for participation in Phase II CCEP associated with serostatus, Army Gulf War Veterans Mycoplasma Study*

Model	Factors in the model	Pre- and post-deployment serostatus	Odds ratio	95% CI
1	Age, gender, specimen time interval, days in Gulf, race, marital status, rank, component	Negative (0–0)	1·00	
		Seroconversion (0–1)	1·12	(0·32, 3·97)
		Seropositive (1–1)	0·98	(0·38, 2·57)
		Seroreversion (1–0)	1·08	(0·42, 2·78)
2	Age, race, marital status, rank, component	Negative (0–0)	1·00	
		Seroconversion (0–1)	1·14	(0·32, 4·03)
		Seropositive (1–1)	0·95	(0·37, 2·48)
		Seroreversion (1–0)	1·08	(0·42, 2·80)

Table 5. *Logistic regression models for participation in Phase II CCEP associated with serostatus, Army Gulf War Veterans Mycoplasma Study: Regular Army only*

Model	Factors in the model	Pre- and post-deployment serostatus	Odds ratio	95% CI
1	Age, gender, specimen time interval, days in Gulf, race, marital status, rank	Negative (0–0)	1·00	
		Seroconversion (0–1)	1·15	(0·32, 4·08)
		Seropositive (1–1)	0·96	(0·36, 2·59)
		Seroreversion (1–0)	1·03	(0·40, 2·69)
2	Age, race, marital status, rank	Negative (0–0)	1·00	
		Seroconversion (0–1)	1·19	(0·34, 4·20)
		Seropositive (1–1)	0·93	(0·35, 2·50)
		Seroreversion (1–0)	1·02	(0·39, 2·67)

(95% CI = 0·32, 4·08); Model 2: OR = 1·19 (95% CI = 0·34, 4·20)].

DISCUSSION

Mycoplasma, particularly *M. fermentans*, has been proposed as a likely cause of GWI among veterans who were deployed in ODS based on a technique called NGT (15–17). The hypothesis has received much attention among veterans, the press and the scientific community. To test the *M. fermentans*–GWI hypothesis, we performed a case-control study to test for *M. fermentans*-specific antibodies using ELISA/WB in a group of Gulf War veterans self reporting various clinical symptoms that have been collectively referred to as GWI and in a group of veterans who did not participate in the registry for clinical evaluation. We found, based on a large sample size, no serological evidence that infection by *M. fermentans* is associated with veterans who have GWI. The prevalence of seropositivity for *M. fermentans* was low in both cases and controls before deployment (4·8 *vs.* 5·2%). There was no difference in rates of seroconversion, from negative to positive antibodies to *M. fermentans*, during this period among cases and controls (1·1 *vs.* 1·2%) that were deployed to ODS. These findings

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suggest that infection by *M. fermentans* is unlikely to play an important role in the disease process currently being called GWI in the ODS veterans.

It is interesting to note that 17 (2·4%) of 718 individuals in the case group and 62 (2·8%) of 2233 individuals in the control group seroreverted from positive to negative test results during the study period. Although the actual mechanism of seroreversion in these individuals is not clear, it is likely that many could have received treatments of antibiotics for various infections during these 3·5 years. Antibiotic treatment or the host immune response might have cleared the mycoplasmal organisms followed by loss of detectable antibodies. Statistically significant differences were not found for the seroreversion between cases and controls.

Our results are in sharp contrast to the earlier studies based on NGT test of *M. fermentans*. Clearly, two completely different techniques based on distinct diagnostic principles were used to examine possible infection by *M. fermentans* in veterans with GWI. The NGT study was designed to detect hybridization signal of *M. fermentans* DNA in nuclear fractions from blood cells of patients [15–17]. Our study is based on finding specific antibodies produced by the host immune system in response to the mycoplasmal infection. We previously showed mycoplasma-specific antibodies are produced in most patients with AIDS despite having severely compromised immune functions [18–22]. For example, all the HIV-infected patients who tested positive by polymerase chain reaction (PCR) for *M. genitalium* were found to be positive in ELISA testing of *M. genitalium*-specific antibodies [22]. Thus, we expected to find the frequency of *M. fermentans*-specific antibodies in Gulf War veterans with GWI to be extremely high, given that more than 50% of veterans with GWI are indeed infected by *M. fermentans* as described by the NGT study. We also expected seroconversion among veterans with GWI would be significantly higher than those who did not enroll in the registry.

It has been argued that NGT, a technique that does not amplify any specific sequence, is more sensitive than PCR in detection of mycoplasmal infection [15–17]. However, it is difficult to assess the validity and specificity of NGT testing, as there is no precedent for identifying any viral, mycoplasmal or bacterial infection in clinical specimens using this uncommon technique. In our laboratory, a PCR study of peripheral blood, urine and oral swabs from veterans with or without complaint of 'GWI', using *M. fermentans*-specific insertion-like sequence [24, 25] showed extremely low positivity rates in both groups (unpublished data).

As stated in the Introduction, GWI still lacks a well-defined case definition with characteristic physical signs, symptoms or laboratory abnormalities. In this study, the cases were participants of the second phase of clinical evaluation for various unexplained illnesses in CCEP. A previous study showed that only 12% of the original 20000 ODS veterans who participated in the initial voluntary health programme advanced to phase II evaluation [12]. The exclusion criteria for the controls were either veterans who did not participate in the ODS or ODS veterans who believed he or she had a health concern and requested a medical evaluation. Some individuals in the randomly selected matched controls might have some of the similar, often non-specific, symptoms reported by the cases but chose not to enroll in the registry for unknown reasons. However, finding a low level of seropositivity and seroconversion for *M. fermentans* in both the case and the control groups very much excludes *M. fermentans* as a possible cause of GWI.

Although our current study provides direct evidence arguing against *M. fermentans* as a probable cause of GWI, this finding does not preclude the possibility that GWI may have an infectious aetiology including a yet unidentified human mycoplasma. There is little doubt that many GW veterans are ill. Moreover, there is a marked difference between GW veterans and non-deployed veterans in the reporting of various clinical symptoms [13]. An infectious organism could play an important role in the pathogenesis of at least a portion of the GW veterans who often present with a heterogeneous spectrum of unexplained illnesses. In this context, the numerous accounts of veterans reporting clinical improvement following treatment by antibiotics that are effective against mycoplasmas (and many other prokaryotic microorganisms) [14–17] may have a scientific basis. However, only a carefully controlled, double-blinded study could ultimately show whether there is a true improvement in veterans with GWI, rather than a common placebo effect following antimicrobial treatments.

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