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# Positive correlation between serum immunoreactivity to *Demodex*-associated *Bacillus* proteins and erythemato-telangiectatic rosacea

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## Summary

*Background* Rosacea is a chronic inflammatory condition that affects the skin of the face and the eyes. Erythematotelangiectatic rosacea is characterized by flushing, oedema and telangiectasia. Patients with rosacea demonstrate elevated densities of *Demodex* mites in their skin compared with controls. A bacterium (*Bacillus oleronius*) isolated from *Demodex* mites from a patient with papulopustular rosacea has been demonstrated to produce antigenic proteins that may play a role in papulopustular and ocular rosacea.

*Objectives* To establish whether there was a correlation between the reactivity of sera from patients with erythematotelangiectatic rosacea to *Bacillus* antigens, and to characterize the proteins to which these patients showed reactivity.

*Methods* Serum samples from patients with erythematotelangiectatic rosacea and controls were examined for reactivity to *Bacillus* proteins by Western blot analysis. Proteins to which the sera reacted were excised from gels, trypsin digested, and putative identities were assigned following liquid chromatography-mass spectrometry (LC-MS) analysis.

*Results* Eighty per cent (21/26) of patients with erythematotelangiectatic rosacea showed serum reactivity to the 62- and 83-kDa proteins of *B. oleronius*, compared with 40% (9/22) of controls ($P = 0{\cdot}004$). The 62-kDa protein was characterized by LC-MS and showed homology to groEL chaperonin, which provokes a strong immune response in mammals. The 83-kDa protein showed homology to aconitate hydratase, of which expression is increased in bacteria under oxidative stress, and which is highly immunogenic.

*Conclusions* The majority of patients with erythematotelangiectatic rosacea show serum reactivity to two proteins from *B. oleronius*, suggesting that this bacterium may play a role in the induction of this condition. The two proteins to which patient sera reacted were found to be similar to a heat shock protein and an enzyme involved in regulating the stress response of the bacterium.

Rosacea is a chronic inflammatory condition of the skin of the face.[1] A formal classification of the disease has been developed and recognizes a number of subtypes including papulopustular rosacea (PPR) and erythematotelangiectatic, ocular and phymatous rosacea.[2] Although frequently encountered by dermatologists, rosacea is a complex disease and a variety of factors, including environmental factors (weather, spicy foods, stress or physical exertion),[3] alterations in the innate immune response, vascular changes in the skin and the presence of reactive oxygen species within the skin, may contribute to the induction and persistence of the condition.[3–5] Erythematotelangiectatic (type 1) rosacea is characterized by persistent flushing in the central regions of the face, accompanied by oedema and telangiectasia.[2,5,6] Patients may experience stinging and burning sensations and have extremely sensitive skin that can be rough or scaly. Many patients with erythematotelangiectatic rosacea may simultaneously experience symptoms of PPR and/or ocular rosacea.[5]

Light therapy (lasers or intense pulsed light devices) can be effective in the treatment of erythematotelangiectatic rosacea, and pulsed dye laser (PDL) is often the most effective.[7] PDL causes photothermolysis, thus inducing selective coagulation of the superficial blood vessels without further damage. This results in a significant reduction of telangiectasia and erythema, but also

induces a reduction in the burning sensation and stinging by lowering the concentration of the mediators released, especially the neurotransmitter substance P.[8] A major concern when using this therapy for the treatment of erythematotelangiectatic rosacea is the generation of purpura, a discoloration caused by bleeding under the skin surface after treatment, and the fact that it has limited efficacy for vessels larger than 0·2 mm in diameter and located more than 1 mm from the skin surface.[7,9]

Patients with rosacea demonstrate an increased density of *Demodex* mites compared with unaffected controls.[10–12] These mites can be either *Demodex folliculorum* or *Demodex brevis*, and inhabit the pilosebaceous unit where their food source may be sebum and/or protein. The nature of the lipids in sebum from patients with PPR is different from that in sebum from controls, and thus may facilitate the growth and survival of mites.[13] The significance of the elevated *Demodex* density in patients with rosacea has been the subject of much speculation. We previously isolated a bacterium (*Bacillus oleronius*) from a *Demodex* mite from a patient with PPR,[14] and demonstrated a strong correlation between patients' serum reactivity to *Bacillus* antigens and PPR[14] and ocular rosacea.[15] Western blot analysis revealed reactivity to two antigenic proteins of size 62 and 83 kDa when probed with sera from patients with rosacea.[14] It was postulated that these antigens, along with others, are released from dead *Demodex* mites and thus lead to an inflammatory reaction around the pilosebaceous unit.[16] The elevated density of *Demodex* mites in the skin of patients with rosacea may lead to the release of high levels of stimulatory antigens and toxins, which leak from the pilosebaceous unit and lead to neutrophil activation and thus stimulate inflammation.

The aim of the work presented here was to examine the reactivity of sera from patients with erythematotelangiectatic rosacea to proteins from *B. oleronius* in order to establish whether there was a similar correlation to that seen between serum reactivity with PPR[14] and ocular rosacea.[15] The demonstration of a role for these bacterial proteins in the erythema and inflammation associated with erythematotelangiectatic rosacea may facilitate the development of more effective and targeted therapies.

## Materials and methods

### Extraction of bacterial antigens

Nutrient broth (250 mL, pH 7) was inoculated with *B. oleronius* and incubated for 48 h at 30 °C and 200 r.p.m. Late stationary phase cells were sedimented by centrifugation at 4000 ***g*** for 20 min. The supernatant was discarded and cells were washed twice with PBS (pH 7·2). Cells were resuspended in lysis buffer containing 0·2% Triton-X 100 with added protease inhibitors (10 μg $mL^{-1}$ each of leupeptin, pepstatin A, aprotinin and TLCK). The suspension was inverted for 1 h at 4 °C before sonication at 20% power for three 10 s blasts using a soniprobe sonicator (Sonopuls HD 2200; Bandelin, Berlin, Germany). The supernatant was centrifuged at 6000 ***g*** at 4 °C for 2 min to separate the protein preparation. Protein concentration was assessed by Bradford assay and the protein was resuspended (1 μg $\mu L^{-1}$) in denaturing sample buffer.

### Patient population

Twenty-six patients (nine men, 17 women), Fitzpatrick skin types II–III, were enrolled in the hospital-based study and the average age was 48·3 years (range 20–83 years). At the time of serum sampling all enrolled patients were considered to have subtype 1 rosacea, following dermatological examination. The criteria used for subtype 1 rosacea assessment were the presence of persistent redness and/or telangiectatic vessels on the face (especially in malar regions) without the presence of papular or pustular lesions. Patients were not receiving antibiotic therapy for their condition but were scheduled for laser therapy. Controls were selected from patients attending hospital who did not show symptoms of rosacea, on the same day that serum samples were taken from the patients with rosacea. A total of 21 controls were enrolled (nine men, 12 women) and the average age was 54·3 years (range 28–80 years). All patients and controls were informed about the nature of the study and were enrolled once they agreed to participate. Ethical permission was granted for this study by Centro Hospitalar de Vila Nova Gaia/Espinho, Portugal.

### Western blot analysis using patient sera

Western blots utilized 12·5% (w/v) sodium dodecylsulphate polyacrylamide gel electrophoresis performed in a discontinuous buffer system, in which each well contained 15 μg of *B. oleronius* protein extract. Following electrophoresis the protein was transferred to a nitrocellulose membrane, which was cut into strips for the testing of individual patient sera. A 5 mL sample of peripheral blood was drawn from each patient and the serum was separated from the blood before being stored at −80 °C. Each serum sample was diluted 1/100 (v/v) with the antibody-diluting buffer (50 mmol $L^{-1}$ Tris–HCl, 150 mmol $L^{-1}$ NaCl) containing 0·05% (v/v) Tween-20, 1% (w/v) bovine serum albumin (BSA), and 3% (w/v) nonfat dried milk, and used as the primary antibody. The secondary antibody was antihuman IgG-horseradish peroxidase-linked whole antibody (Sigma Aldrich Chemical Co. Ltd, Poole, U.K.), which was diluted 1/1000 with the antibody-diluting buffer. The immunoreactive protein bands were visualized by incubating the membranes for 10 min in 10 mg of diaminobenzidine tetrahydrochloride in 15 mL of 100 mmol $L^{-1}$ Tris–HCl (pH 7) containing 15 μL of hydrogen peroxide before washing in distilled water and drying. Western blot analysis was performed without prior knowledge of the rosacea status of the patient or control.

### Liquid chromatography-mass spectrometry analysis of bacterial proteins

Gel pieces corresponding to protein bands to which sera reacted were excised and trypsin digested as described previously.[17] Following peptide extraction, identification was

performed using an Agilent 6340 Ion Trap liquid chromatography - mass spectrometry (LC-MS) (Agilent Technologies, Wokingham, U.K.) calibrated using BSA. The resulting mass lists were BLAST searched using the MASCOT MS/MS ion search program (Matrix Science, Boston, MA, U.S.A.) available at http://www.matrixscience.com. Annotated function and protein information was ascertained through the UNIPROT database (http://www.uniprot.org). A Z score greater than 68 is considered significant at $P < 0{\cdot}05$.

LC-MS analysis was employed previously to characterize the range of proteins released by *B. oleronius* that may provoke an immune reaction.[16] It was demonstrated that a number of immunogenic proteins (e.g. peptidoglycan synthesis protein, flagellin, alkyl hydroperoxide reductase and catalase) were released from *Bacillus* cells and could play a role in triggering an immune reaction (e.g. neutrophil migration, cytokine production, degranulation) in and around the pilosebaceous unit, *in vivo*.[16]

### Statistics

A $\chi^2$ test was used to establish the significant difference between the reactivity of the patient and control sera to *Bacillus* proteins.

## Results

### Response of serum from patients with rosacea to *Bacillus* antigens

Serum from patients with rosacea, and age- and sex-matched controls, was isolated and used to probe membranes containing *Bacillus* proteins. Western blots were developed and the reactivity of each serum sample to the 62- and 83-kDa *Bacillus* proteins was examined. The results revealed that 80% (21/26) of patients with rosacea showed serum reactivity to the *Bacillus* proteins, while serum from 40% (9/22) of control patients showed reactivity ($P = 0{\cdot}004$, Fig. 1). The majority of patients with rosacea showed serum reactivity to the 62-kDa protein (85·7%, 18/21), nine patients (42%) showed reactivity to the 83-kDa protein and six patients (28·5%) demonstrated reactivity to both proteins simultaneously. Of controls who showed reactivity to the *Bacillus* antigens, 89% (8/9) reacted to the 62-kDa antigen, 22% showed serum reactivity to the 83-kDa antigen and 11% (1/9) showed reactivity to both antigens simultaneously.

### Characterization of the 62- and 83-kDa proteins by liquid chromatography-mass spectrometry analysis

In the current work the two proteins that patient sera reacted with (i.e. the 62- and 83-kDa proteins) were excised and processed for identification by LC-MS analysis as described (Fig. 2). The results identified the 62-kDa protein as showing homology to a heat shock protein, groEL chaperonin (Table 1). This protein is highly immunogenic and provokes a strong immune response in mice[18] and humans.[19] Bacterial heat shock proteins are known to have antigenic properties, and proteins homologous to groEL have been identified in *Escherichia coli*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* and *Burkholderia pseudomallei*.[20,21] The 83-kDa protein was identified as showing homology to aconitate hydratase (Table 1). This protein is increased in expression during periods of oxidative stress in bacteria and is highly immunogenic.[22] These results

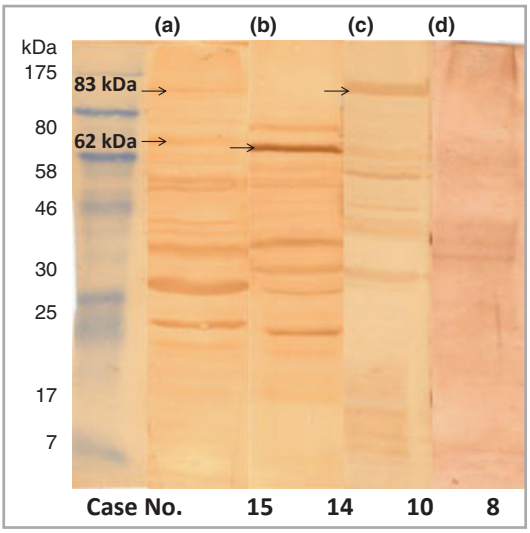


**Fig 1.** Western blot analyses of patient and control sera. Representative examples: (a) positive serum immunoreactivity to both 83- and 62-kDa protein bands (marked by arrows, case no. 15); (b) positive to the 62-kDa protein band (case no. 14); (c) positive to the 83-kDa protein band (case no. 10); (d) negative to both protein bands (case no. 8).

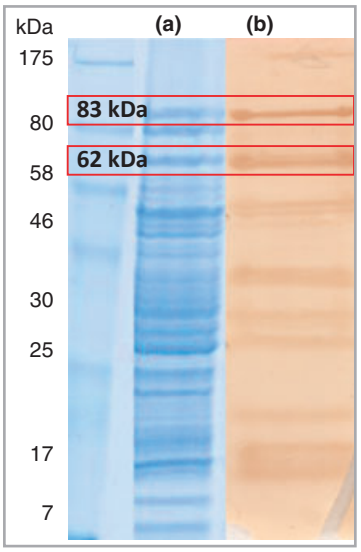


**Fig 2.** (a) *Bacillus oleronius* proteins separated by sodium dodecylsulphate polyacrylamide gel electrophoresis. (b) Western blot analyses of patient sera highlighting the 83- and 62-kDa immunoreactive protein bands.

**Table 1** Liquid chromatography-mass spectrometry identification of immunoreactive protein bands from *Bacillus oleronius*

| ID | Protein | Species | Accession no. | Mass (Da) | pI | Z-score | Coverage |
|---|---|---|---|---|---|---|---|
| 62 | groEL chaperonin | *Bacillus subtilis* | 1906220B | 57 470 | 4·75 | 375 | 15% |
| 83 | Aconitate hydratase | *Bacillus* sp. *SG-1* | ZP_01858967 | 99 213 | 4·94 | 358 | 9% |

indicate that the two proteins to which sera from patients with rosacea react are highly immunogenic and may play a role in triggering an immune response and thus inducing inflammation and tissue degradation.

## Discussion

Rosacea is a complex disease that may involve environmental, genetic, immunological and microbiological components.[2–5] The significance of the elevated *Demodex* density on the faces of patients with rosacea has been the subject of much speculation, and its role in the induction of the disease is not defined.[10–12] There is increasing evidence to suggest a potential role for bacteria in the induction and persistence of this condition. Antibiotics used to treat rosacea suggest a potential role for bacteria in the induction of this condition, although such antibiotics may also display anti-inflammatory properties.[3] *Staphylococcus epidermidis* samples isolated from patients with rosacea (but not controls) were consistently beta-haemolytic and grew better at 37 °C than at 30 °C, suggesting a link between mesophilic hydrolytic bacteria and rosacea.[23] In addition this bacterium has been isolated from the pustules of patients with PPR and from the eyelids of patients with ocular rosacea, indicating a potential role in the induction of these conditions.[24] Previous work has indicated a positive correlation between the reactivity of serum from patients with rosacea to *B. oleronius* proteins and the occurrence of PPR[14] and ocular rosacea,[15] thus suggesting a possible role for this bacterium in the induction of these conditions. The two proteins that patient sera react with were identified as groEL chaperonin and aconitate hydratase. Both of these are highly immunogenic,[18,19,22] and their release from bacteria within *Demodex* mites within the pilosebaceous unit could lead to the initiation of an immune reaction and inflammation in that vicinity. This bacterium has also been recorded in *Demodex* mites from patients with chronic blepharitis, and it was suggested that the *Bacillus* played a role in the development of some forms of this condition.[25]

It has been demonstrated previously that antigens released from *B. oleronius* are capable of activating neutrophils and so could play a role in provoking inflammation around the pilosebaceous unit.[16] *B. oleronius* may be an endosymbiont of *Demodex* mites and may facilitate the digestion of food as they do in the termite.[26] The release of antigens from this bacterium and other bacteria from dead *Demodex* mites in the pilosebaceous unit may cause the activation of neutrophils in the vicinity of the unit and thus explain why in some cases of rosacea the inflammation is centred on the pilosebaceous unit.

We have previously demonstrated that 29% of controls show serum reactivity to the *Bacillus* antigens.[14] In the current study 40% of controls demonstrated serum reactivity to the 62- and 83-kDa proteins of *B. oleronius*. We speculate that *B. oleronius* is a normal part of the flora of *Demodex* mites, but that the low numbers of mites in controls may indicate a low density of bacteria and bacterial proteins released upon the death of mites, which may not be sufficient to provoke a significant immune response. The elevated density of mites in patients with rosacea,[18] and consequently their attendant bacteria, may result in the release of large amounts of stimulatory *Bacillus* proteins that leak from the pilosebaceous unit and provoke an immune response.[16]

Erythematotelangiectatic rosacea is characterized by erythema, oedema and telangiectasia.[2,5,6] Antibiotic therapy may be used to control the condition,[3] suggesting a possible role for bacteria in its aetiology. We postulate that these antibiotics kill the *Bacillus* within the *Demodex* mites, and thus no new stimulatory proteins are released. The loss of the endosymbiotic bacteria would adversely affect the metabolism of the *Demodex* mites and their numbers would decline. A resurgence in the *Demodex* population could occur after the cessation of antibiotic therapy, thus leading once again to an increase in the population of *B. oleronius* in the face, and the reactivation of symptoms. The identification of a role for bacteria in rosacea may facilitate the development of more effective therapies that target the bacterial population within *Demodex* mites.

### What's already known about this topic?

- Patients with rosacea demonstrate a high density of *Demodex* mites in the skin, but the role of these or associated microbes in the induction of the condition is unclear.

### What does this study add?

- Patients with erythematotelangiectatic (type 1) rosacea demonstrate a strong serum reactivity to proteins from *Bacillus oleronius*, which was originally isolated from a *Demodex* mite from a patient with rosacea.
- This result suggests that immune reactivity to the *Bacillus* proteins may have a role in the induction of this form of rosacea.

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