Journal of Medical Microbiology (2014), **63**, 258–262 DOI 10.1099/jmm.0.065136-0

Correlation between serum reactivity to *Demodex*-associated *Bacillus oleronius* proteins, and altered sebum levels and *Demodex* populations in erythematotelangiectatic rosacea patients

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Rosacea is a chronic inflammatory condition that affects the skin of the face and the eyes. The aetiology of rosacea is not clearly established but increasing evidence suggests a potential role for bacteria in the induction of the condition. A role for *Bacillus oleronius*, originally isolated from within a *Demodex folliculorum* mite, in the aetiology of the condition has been suggested. The aim of the study was to determine whether a correlation existed between the level of sebum and the density of *D. folliculorum* in the skin of erythematotelangiectatic rosacea patients, and the reactivity of these patients' sera to proteins of *B. oleronius*. Serum reactivity to the 62 and 83 kDa *B. oleronius* proteins was found in 82.6 % (62/75) of the rosacea patients and in 26.9 % (14/52) of controls (P=0.0016). In the group of rosacea patients whose sera reacted to *B. oleronius* proteins, the level of sebum was statistically lower than in controls (P=0.01). The density of *D. folliculorum* on the face of *Bacillus* positive rosacea patients was statistically higher than controls (P=0.0001). Rosacea patients demonstrated increased *Demodex* populations on their faces and reduced sebum levels. Their sera also showed reactivity to *B. oleronius* proteins, suggesting a potential role for this bacterium in the aetiology of rosacea.

Received 8 June 2013
Accepted 15 November 2013

INTRODUCTION

Rosacea is a common, chronic, multiphase inflammatory dermatosis of the face, the course of which is characterized by periods of exacerbation and remission (Wilkin *et al.*, 2002). The most frequent skin changes in rosacea patients include flushing or permanent erythema, papules, pustules and telangiectasias, located in the central part of the face i.e. cheeks, nose, chin and forehead (Wilkin *et al.*, 2002; Crawford *et al.*, 2004). Four basic subtypes of rosacea have been identified: erythematotelangiectatic, papulopustular, phymatous and ocular (Wilkin *et al.*, 2002). Erythematotelangiectatic rosacea is characterized by extensive erythema and oedema on facial skin (Wilkin *et al.*, 2002) and may be controlled by the use of selected antibiotics or by pulsed dye laser (PDL) therapy (Gupta & Chaudhry, 2005). However, the inflammation associated with papulopustular rosacea tends to be centred on the pilosebaceous unit (Jarmuda *et al.*, 2012; Holmes, 2013).

Abbreviation: PPR, papulopustular rosacea.

Various factors such as vascular and immunological abnormalities, agents responsible for degradation of the structures of connective tissue and selected infectious causes are believed to play a role in the aetiology of rosacea (Gupta & Chaudhry, 2005; Yamasaki & Gallo, 2009). Since the aetiology of the disease remains unclear, the treatment of rosacea presents a challenge to the clinician and requires a highly individual approach. Management with antibiotics, mostly from the group of tetracyclines, macrolides and metronidazole, is generally recommended (Pelle *et al.*, 2004).

The possible role of *Demodex folliculorum* mites in the pathogenesis of rosacea, especially the mechanism of passive transfer of other micro-organisms, has been speculated upon for many years (Jarmuda *et al.*, 2012). The incidence of *Demodex* on the facial skin of patients with rosacea is significantly higher than in controls (Bonnar *et al.*, 1993). A significantly greater density of the mites per cm^2 was detected in patients with papulopustular rosacea (PPR) (Bonnar *et al.*, 1993) and the composition of the lipids from

their sebum revealed differences in comparison with controls, which might facilitate the development of larger populations of mites (Ní Raghallaigh *et al.*, 2012). The presence of *D. folliculorum* in the sebum secretions from the pilosebaceous unit was found in 90.2 % of PPR patients and only in 11.9 % of healthy controls. Additionally, histological tests of skin samples obtained from these patients revealed that the presence of *Demodex* was strongly correlated with substantial perifollicular lymphocytic infiltration (Georgala *et al.*, 2001). *Bacillus oleronius* was successfully isolated from a *Demodex* mite obtained from a PPR patient (Lacey *et al.*, 2007), where it may play a role in facilitating digestion, as it does in the termite (Kuhnigk *et al.*, 1995). This bacterium produced two highly immunogenic proteins that showed reactivity to sera from PPR (Lacey *et al.*, 2007), ocular (Li *et al.*, 2010) and erythematotelangiectatic (O'Reilly *et al.*, 2012c) rosacea patients. It has been suggested that the release of *B. oleronius* proteins from dead *Demodex* mites may lead to neutrophil recruitment and activation in the vicinity of the pilosebaceous unit (O'Reilly *et al.*, 2012a), thus possibly explaining why the inflammation in rosacea is often centred around this structure. The potential role of these bacterial proteins in inducing corneal damage in ocular rosacea has been described (O'Reilly *et al.*, 2012b).

The aim of the work presented here was to establish whether a correlation existed between the sebaceous condition of the skin, the density of *Demodex* mites and reactivity of sera obtained from rosacea patients to *B. oleronius* proteins, in order to determine the role of *B. oleronius* in the induction of this disfiguring condition.

METHODS

Study population. Seventy-five patients with erythematotelangiectatic rosacea (33 males and 42 females), Fitzpatrick skin phototypes I or II, aged 20–81 years, hospitalized between 1 February 2011 and 16 December 2011 at the Dermatology Clinic, Poznań University of Medical Sciences or treated at the out-patient Dermatology Clinic, were enrolled in the study. Mean age of rosacea patients was 47.07 years (females, 44.95; males, 49.76). Patients did not receive any oral antibiotics, retinoids, glucocorticosteroids or sulfones for at least 3 months prior to recruitment to the study.

Fifty-two volunteers (28 females and 24 males), aged 18–89 years, constituted the control group. The mean age of the controls was 46.26 years (females, 47.45; males, 44.83). The study was approved by the Bioethics Committee at Poznań University of Medical Sciences (546/10, 17 June 2010).

Medical history acquisition, physical examination and additional tests were performed for all patients. Before enrolment, all patients and controls were informed about the nature and the aim of the study and gave their written informed consent. The diagnosis of rosacea was made on the basis of their medical history and physical examination. A standard classifications system, published by the National Rosacea Society (Wilkin *et al.*, 2002), was used in the process of the diagnosis and classification of rosacea.

Plasma samples. Samples of peripheral blood (20 ml) from the cubital vein were collected from all study participants between 8 a.m. and 1 p.m. into EDTA tubes (Monovette, Sarstedt). The blood was centrifuged at 500 ***g*** for 10 min. The serum specimen was separated into three parts and stored at −80 °C.

Preparation of bacterial protein for Western blotting of patient serum samples. *B. oleronius* cells were cultured in nutrient broth to the stationary phase and subjected to cell surface protein extraction using 0.2 % (v/v) Triton X-100 as previously described (Lacey *et al.*, 2007; O'Reilly *et al.*, 2012a). The protein concentration was determined by Bradford assay and protein was resuspended at a concentration of 1 μg μl^{-1} in denaturing sample buffer. *Bacillus* protein (20 μg per well) was separated by 1D SDS-PAGE on 12.5 % acrylamide gels. Following electrophoresis, *Bacillus* proteins were transferred to nitrocellulose membranes which were sectioned into strips. Following a membrane blocking wash, individual serum samples (diluted 1/100 in antibody diluting buffer) were applied overnight at 4 °C. Following a TBS-Tween wash, the secondary anti-human IgG-HRP-linked whole antibody (Sigma) was applied at a dilution of 1/1000 for 2 h at room temperature. Immunoreactive protein bands were visualized by incubating membrane strips in diaminobenzidine tetrahydrochloride [DAB; 1 mg l^{-1} in 100 mM Tris/HCl (pH 7) containing 15 μl hydrogen peroxide] for 10 min at room temperature. All Western blots were performed using blinded serum samples and all were performed on three independent occasions.

Standardized skin surface biopsy (SSSB). One drop of cyanoacrylate adhesive was placed on a glass slide with a pre-marked square surface area of 1 cm^2. The slide was applied to the skin in the central area of the right cheek on a patient's face. After 30 s, the slide was removed gently and one drop of immersion oil was added. A coverslip was placed on the sample and the specimen was examined under an optical microscope (magnified ×40 and ×100). The number of *Demodex* mites per cm^2 was enumerated by microscopic examination.

Sebumetric test. The level of sebum secretion by the skin was measured using a Sebumeter SM 815 Courage-Khazaka (Courage-Khazaka Electronic) as recommended by the manufacturer. A piece of 0.1 mm tape on a measuring probe, equipped with a spring to assure constant and even pressure, was placed on the skin of the centre of the patient's chin for 30 s. The probe was then inserted into the sebumeter, where the amount of sebum on the surface of the skin was measured and expressed as μg cm^{-2}.

Statistical methods. The statistical significance was assessed by the χ^2-test and Student's *t*-test using GraphPad Prism version 5.00 for Mac OS X, GraphPad Software, www.graphpad.com. *P*-values <0.05 were considered statistically significant.

RESULTS

Reactivity of patient sera to *Bacillus* proteins

Protein was extracted from *B. oleronius* cells, resolved by 1D SDS-PAGE and transferred to membranes for Western blotting as described. Serum from patients with erythematotelangiectatic rosacea and controls was isolated and used to probe membranes containing the *Bacillus* proteins. The number of serum samples showing reactivity to the 62 and 83 kDa proteins of *B. oleronius* was calculated for each cohort (Fig. 1). The results revealed that 26.9 % (14/52) of controls showed reactivity to the bacterial proteins while 82.6 % (62/75) ($P=0.0016$) of patients diagnosed with erythematotelangiectatic rosacea showed reactivity to

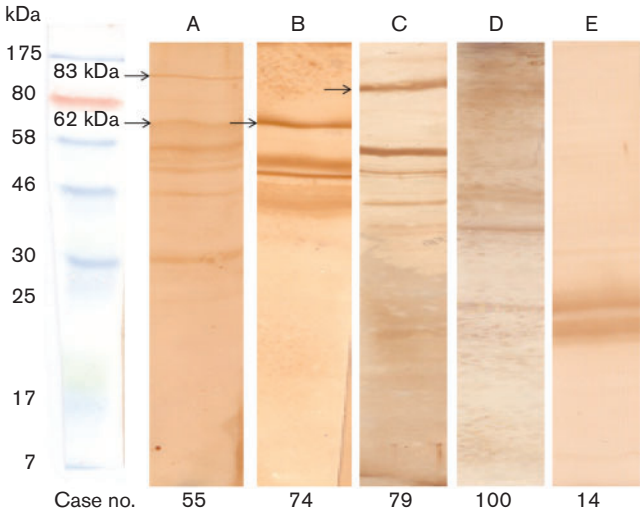

Fig. 1. Representative Western blots of reactivity of rosacea patients and control sera to *B. oleronius* proteins. Positive serum immunoreactivity to (A) both 83 and 62 kDa protein bands (marked by arrows, case no. 55), (B) the 62 kDa protein band (case no. 74), (C) the 83 kDa protein band (case no. 10), (D) negative serum immunoreactivity reactivity to both protein bands (case no. 100) and (E) serum from a control patient negative for reactivity to both bands (case no. 14).

the *Bacillus* proteins (Fig. 2). Rosacea patients could be divided into two groups on the basis of their reactivity (62/75) or non-reactivity (13/75) to the *Bacillus* proteins and were termed *Bacillus* protein reactive or *Bacillus* protein non-reactive, respectively.

Analysis of *Demodex* population in rosacea patients and controls

Analysis of the *Demodex* population in the skin of rosacea patients and controls revealed a statistically greater number of *Demodex* mites in the skin of rosacea patients that showed reactivity to the *Bacillus* proteins ($P<0.0001$) (Fig. 3). There was a slightly lower, although statistically non-significant ($P=0.559$), *Demodex* population in the skin of *Bacillus* protein non-reactive rosacea patients than in *Bacillus* protein reactive patients.

Bacillus antigen reactive rosacea patients display reduced levels of sebum in their skin

Analysis of the sebum level in the skin of patients and controls demonstrated that *Bacillus* protein reactive rosacea patients showed a lower level of sebum than the controls ($P=0.0013$) (Fig. 4). There was no significant difference between the sebum level in control and *Bacillus* protein non-reactive rosacea patient sera ($P=0.548$). Interestingly, the *Bacillus* protein reactive rosacea patient sera showed a significantly lower level of sebum than the antigen non-reactive rosacea patients ($P=0.0159$).

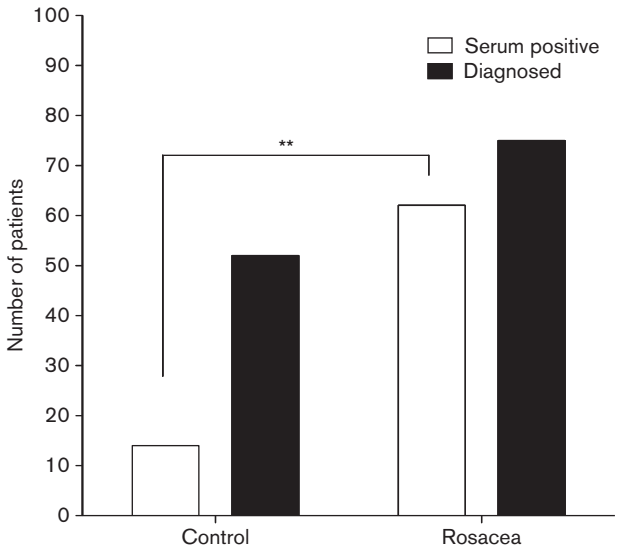

Fig. 2. Rosacea patient and control sera reactivity to 62 and 83 kDa proteins of *B. oleronius*. Reactivity of patient sera to 62 and 83 kDa proteins of *B. oleronius* was recorded by Western blot. Open symbols indicate patients or controls, and closed symbols indicate those showing reactivity to *B. oleronius* proteins. $^{**}P=0.0016$.

DISCUSSION

The results presented here indicate that sera from 82.6 % of erythematotelangiectatic rosacea patients reacted with the 63 and/or 82 kDa protein(s) of *B. oleronius*. In addition, these patients displayed a higher population of *Demodex* mites in their skin and a lower level of sebum than controls.

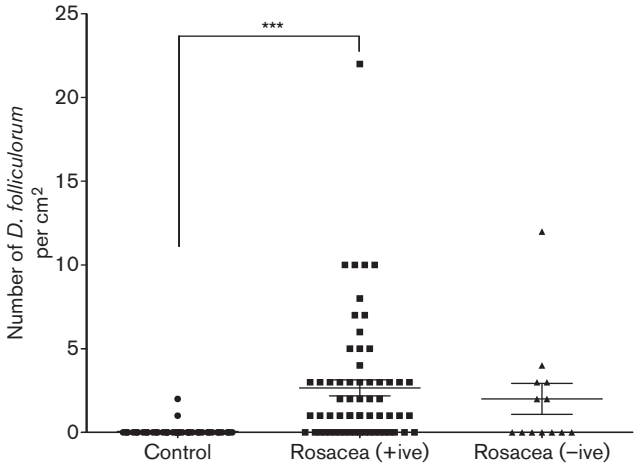

Fig. 3. Variations in *Demodex* population on faces of rosacea patients and controls. *Demodex* mites were extracted from the skin of patients and controls and enumerated as described. Rosacea (+ive) indicates rosacea patients who were reactive to the *Bacillus* proteins; Rosacea (−ive) indicates rosacea patients who did not react to the *Bacillus* proteins. $^{***}P<0.0001$.

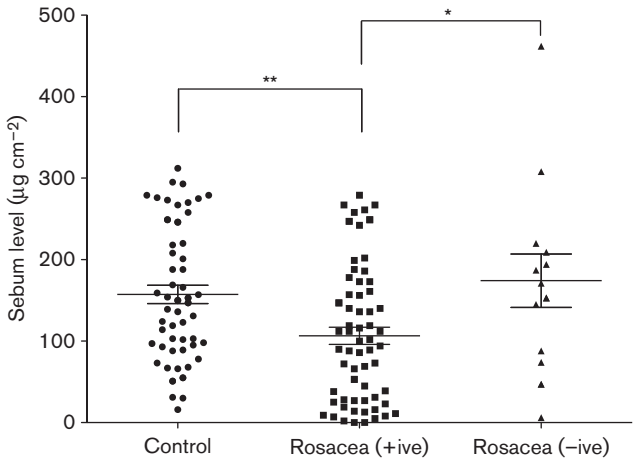

Fig. 4. Variation in sebum level on skin of rosacea patients and controls. The sebum level on the skin of rosacea patients and controls was measured and expressed as μg cm^{-2}. Rosacea (+ive) indicates rosacea patients who were reactive to the *Bacillus* proteins; Rosacea (−ive) indicates rosacea patients who did not react to the *Bacillus* proteins. *P=0.0159, **P=0.0013.

A possible role for micro-organisms in the aetiology of rosacea has been the subject of significant debate (Li *et al.*, 2010; Jarmuda *et al.*, 2012). Investigators have attempted to uncover the significance of the increased density of *Demodex* mites on the facial skin of rosacea patients and their role in the pathogenesis of the disease (Erbağci & Ozgöztaşi, 1998; Yamasaki & Gallo, 2009). One of the suggested pathogenic mechanisms is connected with the fact that *Demodex* mites may transmit various bacteria. This theory is supported by the effectiveness of the antibiotic treatment (e.g. doxycycline, minocycline tetracycline), although these antibiotics may also function as anti-inflammatory agents (Gupta & Chaudhry, 2005).

B. oleronius, originally isolated from a *D. folliculorum* mite from a PPR patient, may play a role in the development of dermatological changes associated with rosacea (Lacey *et al.*, 2007). Two proteins derived from *B. oleronius* (62 and 83 kDa) were isolated and their highly immunogenic properties were demonstrated. Exposure of neutrophils to *B. oleronius* proteins leads to their activation and triggers the release of matrix metallopeptidase 9 (MMP-9) and cathelicidin. The stimulation of IL-8 and TNF-α production may result in the development of an inflammatory process *in vivo* (O'Reilly *et al.*, 2012a). The exposure of neutrophils to immunogenic proteins may occur in and around the pilosebaceous unit, when the proteins of *B. oleronius* are released from the dead *Demodex* mites (O'Reilly *et al.*, 2012a). As a consequence of the inflammatory process, the structures of the tissue surrounding the pilosebaceous unit may be damaged (Kafienah *et al.*, 1998).

The release of *B. oleronius* antigens may explain neutrophil activation around the pilosebaceous unit and the fact that the inflammatory process does not extend beyond this area. It is possible that exposure to low levels of *Bacillus* protein in normal skin does not sufficiently challenge the immune response but that the large amounts of material released within the pilosebaceous unit in rosacea patients may induce neutrophil migration and activation (O'Reilly *et al.*, 2012a). Antibiotics that are commonly used in the treatment of rosacea do not reduce the population of *Demodex* but inhibit the growth of *B. oleronius* (Lacey *et al.*, 2007) and thus may prevent the release of *Bacillus*-associated immunogenic proteins into and around the pilosebaceous unit. After antibiotic therapy is discontinued, rosacea symptoms may return, possibly due to the gradual revival of the *B. oleronius* population in the digestive system of the *Demodex* mites (Jarmuda *et al.*, 2012).

The revival of the *B. oleronius* population is possible due to the fact that this bacterium, like other members of the *Bacillus* family, exists in two possible stages: vegetative and endosporic (Szkaradkiewicz *et al.*, 2012). *Demodex* mites migrate on the surface of the host skin and most probably feed on the sebum components and epithelial cells. *B. oleronius* endospores enter their digestive systems in the process and germinate into their vegetative forms. PPR patients have higher pH and reduced levels of hydration of their facial skin (Ní Raghallaigh & Powell, 2009). They also display a different composition of fatty acids in the sebum, with elevated levels of myristic acids and reduced levels of specific saturated fatty acids (Ní Raghallaigh *et al.*, 2012). Perhaps such conditions, not necessarily connected with the levels of sebum but rather with its composition, create a favourable environment for the development of the mite population.

While the aetiology of rosacea is unclear, dermatological, immunological, microbiological and environmental components probably contribute to its appearance (Gupta & Chaudhry, 2005; Yamasaki & Gallo, 2009). Changes to the skin such as an alteration in the amount and composition of sebum (Ní Raghallaigh *et al.*, 2012) may favour the growth of the *Demodex* population. The elevated population of *Demodex* mites in the pilosebaceous unit of rosacea patients may physically distend the unit and facilitate the exit of bacterial proteins and toxins, which might leak into the surrounding tissue and attract neutrophils (O'Reilly *et al.*, 2012a). This scenario might explain why inflammation is often centred on the pilosebaceous unit in rosacea. Treatment of rosacea with antibiotics destroys the *Bacillus* population within the *Demodex* mite and thus prevents the release of additional proteins. Once antibiotic therapy ends, the remaining mites may encounter *Bacillus* endospores on the skin, which germinate in their digestive tract and allow them to feed upon the altered sebum content of the face, thus leading to the renewal of the release of *Bacillus* proteins and the reappearance of symptoms.

Alterations in the nature of the sebum produced in the face may facilitate the increase in the density of *Demodex* in the

skin of rosacea patients. These may release bacterial antigens upon their death in and around the pilosebaceous unit, and the antigens of *B. oleronius* have been shown to induce an inflammatory reaction (O'Reilly *et al.*, 2012a). This scenario would implicate bacteria as having a key role in the induction of rosacea, but this role might only come into play once other factors (e.g. vascular damage, altered sebum, increased *Demodex* density) have occurred. A clear understanding of the factors that contribute to the aetiology of rosacea will assist in the development of more effective therapies for the control of this chronic, disfiguring condition.

ACKNOWLEDGEMENTS

F. M. and A. H. are funded by the Programme in BioAnalysis and Therapeutics (BioAT) by the Higher Education Authority of Ireland through PRTLI V. The authors have no conflicts of interest to declare.

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