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(54) Title: A METHOD FOR DIAGNOSING PROSTATIC DISEASE

(57) Abstract: The present invention provides a method for diagnosing prostatic disease and/or risk of prostatic disease in men comprising obtaining a body fluid sample from a patient and assaying for the presence of antibodies specific for *Propionibacterium acnes (P. acnes)* in the body fluid sample.

A method for diagnosing prostatic disease

FIELD OF THE INVENTION

The present invention relates to methods for diagnosing prostatic disease in men. In particular, the present invention provides a method for diagnosing prostatic disease and/or the risk of prostatic disease in men by assaying for the presence of antibodies specific to *P. acnes*.

BACKGROUND OF THE INVENTION

The inventors have previously cultured *Propionibacterium acnes* (*P. acnes*) from the prostate tissue of a considerable proportion of prostate cancer patients and shown a statistically significant association between positive culture of this bacterium and increased levels of inflammation in the prostate tissue [1]. The inventors have also showed that the majority of *P. acnes* in the prostate gland [1] and in the urinary tract of adult males [2] are types IB and II, which differ genetically and phenotypically from the common skin *P. acnes* of type IA [1-4]. Chronic inflammation is strongly implicated in the development of prostate cancer and other prostate diseases [5] and the inventors' findings suggest that *P. acnes* (in particular types IB and II) may be involved in the development of these prostate diseases through an inflammatory mechanism. *P. acnes* is known to be a potent inflammatory stimulus to the immune system [6] and has been implicated in several other inflammatory conditions including acne vulgaris, endocarditis, endopthalmitis and osteomyelitis [7].

A need exists to develop a non-invasive test allowing diagnosis of *P. acnes* prostatitis in asymptomatic men at a young age prior to the development of benign prostatic hyperplasia or widespread prostatic atrophy, dysplasia and prostate cancer. Various therapies including antibiotics could then be offered to men with positive test results in the hope of preventing a proportion of prostate tumours and other infection-related prostate diseases. However, the finding that *P. acnes*, in particular types IB and II, are common inhabitants of the adult male urethra [2] means that samples such as urine,

ejaculate or prostatic secretions may not be suitable for indirect assessment of the prostate gland due to contamination with the urethral flora during collection. Direct quantitation of *P. acnes* levels by prostatic biopsy is currently too invasive a procedure for testing of asymptomatic men. A need therefore exists to develop an alternative, less invasive approach.

In conditions such as acne vulgaris, research has shown that antibody titres to *P. acnes* are elevated in subjects with severe inflammatory acne [6,8]. This raises the possibility of using immunologic testing for elevated antibody titres against *P. acnes* as a means to identify men with *P. acnes* infection of the prostate gland. However, the general population has extremely high natural antibody titres against *P. acnes*, therefore it is difficult to develop a test which can effectively distinguish patients with *P. acnes* infection. Since the main antigens of *P. acnes* are cell surface and/or secreted carbohydrates [9,10], the excess of the human antibody response might be avoided by purifying out more specific protein antigens for use in immunologic tests. This approach has been used to assess immune response in acne patients, using extracts of the total *P. acnes* cellular proteins or secreted proteins purified from the culture medium [11-13]. Along with secreted proteins, bacterial cell surface proteins are the major bacterial protein targets encountered by the immune system, thus are particularly suitable for use in development of vaccines or immunologic tests [14].

SUMMARY OF THE INVENTION

In a first aspect of the present invention there is provided a method for diagnosing prostatic disease and/or risk of prostatic disease in men comprising obtaining a body fluid sample from a patient and assaying for the presence of antibodies specific for *P. acnes* in the body fluid sample.

In a second aspect of the present invention there is provided a method according to the first aspect of the invention further comprising the step of measuring the level of prostate specific antigen present.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows secreted and membrane-anchored cell surface proteins of *P. acnes* visualized on SDS-PAGE gel and stained with Coomassie Blue.

Figure 2 shows *P. acnes* secreted/cell surface proteins after transfer onto PVDF membrane and staining with Coomassie Blue. All protein bands have transferred well.

Figure 3 shows immunoblot of *P. acnes* secreted/cell surface proteins screened with the serum of 9 prostate biopsy patients. Each set of two lanes represents duplicate samples from a single patient. Only a minority of the protein bands present on the membrane are bound by patient antibodies, and patients show a range of immunoreactivity from strong immunoreactivity (lanes 1, 2 and 8) to weak immunoreactivity (lanes 3-7, 9).

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have developed an immunological test using a mixture of the secreted and cell surface membrane-anchored proteins of *P. acnes*, with particular emphasis on proteins from *P. acnes* types IB and II, and used these proteins to assess prostate biopsy patients for immunoreactivity to *P. acnes*.

In a first aspect of the present invention there is provided a method for diagnosing prostatic disease and/or risk of prostatic disease in men comprising obtaining a body fluid sample from a patient and assaying for the presence of antibodies specific for *P. acnes* in the body fluid sample.

In a preferred embodiment of the present invention the prostatic disease is selected from the group consisting of prostate cancer, prostatitis and benign prostatic hyperplasia.

In a further preferred embodiment of the present invention the body fluid sample is selected from the group consisting of plasma, serum, urine, prostatic secretions and ejaculate.

In a further preferred embodiment the method according to the first aspect of the invention comprises assaying for the presence of antibodies specific for *P. acnes* secreted proteins or cell surface proteins, or both. In a particularly preferred embodiment, the cell surface proteins include membrane-anchored cell surface proteins.

In a further preferred embodiment of the present invention, the secreted and/or membrane-anchored surface proteins include proteins from *P. acnes* type IB and II.

In a further preferred embodiment of the present invention the antibodies are IgA. In yet a further preferred embodiment, the IgA is secretory IgA.

Detecting for the presence of antibodies specific to *P. acnes* proteins can be achieved using routine assays known in the art. For example, the enzyme-linked immunosorbent assay (ELISA) can be used to detect the presence and immunoreactivity of a particular antibody for its antigen by detecting the amount of chromogenic or fluorogenic substrate (hence "enzyme-linked") conjugated to a secondary antibody which binds the antibody-antigen complex.

In a further preferred embodiment of the present invention the method comprises quantitating the antibody titre of the body fluid sample to *P. acnes* protein.

In a second aspect of the present invention there is provided a method according to the first aspect of the invention further comprising the step of measuring the level of prostate specific antigen present.

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following non-limiting examples.

EXAMPLE 1

Extraction of *P. acnes* secreted/cell surface proteins

Extraction of membrane-anchored surface proteins and secreted proteins from *P. acnes* was performed using a previously published method developed for *Listeria monocytogenes* [15].

Methods

P. acnes was grown at 37°C in brain heart infusion broth supplemented with 5% horse serum to late logarithmic growth phase (OD_{600} = 1.9 to 2.0). 200mL was harvested by centrifugation (4000 x ***g*** for 12mins), the cells were then washed in 6mL of phosphate-buffered saline and resuspended by gently pipetting up and down in 1mL of surface protein extraction buffer (1 mL of 1.0 M Tris pH 7.5, 10µL of 0.5 M EDTA, 10 µL of Sigma P8849 protease inhibitor cocktail) to make a thick suspension. After 30mins incubation at 37°C with moderate agitation, the cells were pelleted by centrifuging (4000 x ***g*** for 12 mins) and the supernatant containing the proteins was carefully drawn off into a clean tube. Protein was quantitated using a standard Bradford assay and stored in small aliquots at minus 80°C.

Results

200mL of culture typically yielded protein concentrations of around:

350-450 µg/mL (*P. acnes* type IB)
650-750 µg /mL (*P. acnes* type IA)
850-950 µg /mL (*P. acnes* type II).

EXAMPLE 2

Characterization of *P. acnes* secreted/cell surface proteins by molecular weight

The secreted/cell surface proteins from each *P. acnes* type were compared and characterized by SDS-PAGE analysis using protein molecular weight standards and staining with Coomassie Blue.

Methods

An aliquot of protein mixture containing 180µg of total protein was thawed. After adding one-fifth of a volume of 100% Trichloroacetic acid and vortex mixing, the mixture was cooled at -20° for 5 mins then left at 4°C overnight for protein precipitation to occur. Proteins were pelleted by centrifugation (6000 x ***g*** for 10 mins) then given 4 washes with 500µL of ice-cold acetone (analytical grade), remaining in acetone at 0°C for at least 30 minutes. A large amount of carbohydrates precipitated at this step and were removed along with the acetone supernatant. Finally the protein pellet was dried briefly at 37°C, taking care not to overdry, and pretreated with 22.5 µL of 0.2M NaOH for 5 mins. The protein sample was then resolubilized for 2 hours at room temperature in 200µL of sample buffer (62.5mM Tris-HCL pH 6.8, 2% SDS, 25% glycerol, 0.01% bromothymol blue, with 5% 2-mercaptoethanol added just before use), with frequent mixing by pipette.

After heating at 95°C for 7 mins, the protein was loaded onto a 4-15% polyacrylamide gel (BioRad Readygel) at a concentration of 20µL per lane and run in a mini-Protean 3 electrophoresis cell (BioRad) at 200V in standard running buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3). Proteins were visualized by staining with the BioSafe Coomassie Blue stain (BioRad) according to the manufacturers instructions.

Results

For all three different *P. acnes* types, the mixture yielded at least 25 discrete protein bands ranging in size from 10 kDa to 150 kDa. Protein extractions from *P. acnes* type

IA and IB had a similar pattern whereas the protein extraction from type II differed slightly and showed significantly higher amounts of two large proteins approximately 100kDa and 150kDa in size (Fig 1). These two large proteins were produced by types IA and IB in small amounts only (Figs 1 and 2).

EXAMPLE 3

Testing immune reaction of prostate biopsy patients to *P. acnes* secreted/cell surface proteins by immunoblot

Patient immunoreactivity to *P. acnes* surface proteins was tested initially by transfer of the proteins to a membrane (western blot) followed by screening with patient serum (immunoblot) to identify which specific protein bands were recognized by patient antibodies.

Methods

For immunoblots, 180 µg of total protein was purified by precipitation and run on an SDS-PAGE gel as described in Example 2, except that a 4-15% polyacrylamide gel (BioRad Readygel) with a prep-well format was used. Following electrophoresis the gel was equilibrated for 1 hour in transfer buffer (48mM Tris, 39mM glycine, 0.05% SDS, 20% v/v analytical grade methanol, pH 9.2). PVDF membrane (BioRad Immun-Blot PVDF) was wet in methanol for a few seconds then equilibrated in transfer buffer for 10 mins prior to use. Western blotting was performed in a Mini-Transblot cell (BioRad) for 1 hour and 40 minutes at 100V (constant), after which the membrane was rinsed (3 x 5min with agitation) in de-ionised water and air-dried on Whatman paper overnight. Transfer of proteins was confirmed by use of pre-stained protein molecular weight standards and by Coomassie Blue staining of the gel after transfer.

Immunoblotting was performed with the BioRad Immun-blot Assay kit according to the manufacturers instructions with minor variations as described below. All of the following steps were performed at room temperature, using gentle agitation for steps where the membrane was immersed in liquid. The next morning the membrane was

wet in methanol for a few seconds, equilibrated for 10 mins in high-salt Tris-buffered saline (TBS; 20mM Tris, 500mM NaCl, pH 7.5) then immersed for 1 hour in blocking solution (TBS with 5% w/v BioRad blotting-grade non-fat milk powder). After 10 mins immersion in wash solution (TBS with 0.05% blotting-grade Tween-20) the membrane was placed into a Mini-Protean 2 Multiscreen Apparatus (BioRad) for screening with patient serum. Each channel was loaded with 600 µL of a patient serum diluted 1:1000 in antibody buffer (wash solution containing 1% w/v BioRad blotting-grade non-fat milk powder) and incubated for 2 hours, with mixing by pipette halfway through this time. For this step the negative control lane (no primary antibody) received only antibody buffer. Each channel was then washed 6 X with wash solution and loaded with 600 µL of secondary antibody solution (Goat Anti-Human Ig (H + L) AP conjugate diluted 1:3000 in antibody buffer) for a 2 hour incubation, with mixing by pipette halfway through this time. After washing each channel 6 X with wash solution, the membrane was removed from the multiscreen apparatus and immersed in TBS for 2 X 5 min washes to remove all Tween-20. For colour development the membrane was immersed in colour development solution (2mL of 25X colour development buffer plus 48mL filtered de-ionised water, with addition of colour reagent A (0.5mL) and colour reagent B (0.5mL) just before use) for 30 - 40 minutes. Finally the membrane was rinsed in de-ionised water (2 X 5 mins) and air-dried.

Results

Optimisation

Inclusion of methanol and SDS in the transfer buffer gave good transfer of all protein bands to the membrane for screening (Fig 2). For immunoblotting, a 1:1000 dilution of patient serum allowed discrimination of patients into two groups based on strong or weak immunoreactivity to *P. acnes* surface proteins (Fig 3).

Reaction to the different P. acnes types

Each person screened gave a similar reaction to all three *P. acnes* types, although in most people the reaction to type IA was slightly stronger. In each person screened, the serum antibodies mostly recognized the same-sized protein bands from each of the three *P. acnes* types, suggesting that the most antigenic surface/secreted proteins are produced by all three types. It is therefore impossible to tell which type(s) of *P. acnes* had caused the patient antibody response.

The major antigenic protein band common to all positive serum samples was around 25kDa in size, with other commonly positive bands being 10, 15, 50 and 100 kDa in size (Fig 3).

Patient Analysis

Of the 26 prostate biopsy patients screened by this method, 11 (42%) had strong reactivity to *P. acnes* and the remaining 15 (58%) had weak reactivity. These two groups did not differ significantly in age, prostate volume, percentage with cancer in the biopsy specimens, total amount (mm) of cancer present in the biopsy specimens, or the degree of inflammation in the biopsy specimens. However, the group with strong reactivity to *P. acnes* did have a statistically significantly higher mean serum prostate specific antigen (PSA) level (12.1 ng/mL compared to 6.9 ng/mL in the group with weak reactivity to *P. acnes*, $p = 0.003$).

Since raised PSA levels can be caused by prostatic infection and inflammation [16,17] it is possible that infection of the prostate with *P. acnes* had caused both the raised PSA levels and the high degree of *P. acnes*-specific immune reactivity in this subset of patients. However, the number of cases analyzed is too small for a definite conclusion to be reached and a larger study is needed to either confirm or disprove this trend.

Furthermore, similar high levels of reactivity to *P. acnes* were seen in 2 of the 4 female controls analyzed to date, showing that this high level of antibodies is not specific to

prostate biopsy patients only, it can clearly also reflect exposure to *P. acnes* in other regions in the body.

EXAMPLE 4

Assessment of immune reaction to *P. acnes* surface/secreted proteins by ELISA test

Enzyme-linked immunoabsorbent assay (ELISA) tests can give a more sensitive and quantitative assessment of immune response to an antigen, plus they are less labour intensive than immunoblotting. We therefore developed an ELISA test using the mixture of *P. acnes* surface/secreted proteins. Although we used a mixture of proteins from *P. acnes* type IB and II, the immunoblot results (Example 3) showed that all 3 types of *P. acnes* produce the same main antigenic proteins, thus the ELISA will detect antibodies raised against any *P. acnes* type, it is not specific for types IB and II.

Methods

Each 96-well polystyrene ELISA plate was used to analyse duplicate samples of serum from 4 patients, using 15µg of surface/secreted proteins in total (7.5 µg from *P. acnes* type IB mixed with 7.5 µg from *P. acnes* type II). The proteins were purified by precipitation as described in Example 2, pre-treated with 2µL of 0.2M NaOH for 5 mins then resolubilized in 45 µL of 0.05M carbonate buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) by incubating at room temperature for 2 hours with frequent mixing by pipette. This was stored at 4°C until use later in the day. In the afternoon the protein mixture was diluted to a concentration of 1.5 µg/mL with carbonate buffer and 100µL was added to each well of the ELISA plate except for the negative (no antigen) control wells, which received only carbonate buffer. The ELISA plate was sealed with an adhesive sealing sheet, wrapped in plastic wrap and incubated at 4°C for 16-18 hours (overnight) to allow protein coating of the wells.

All of the following steps were carried out at room temperature, with reagents warmed to room temperature. Except for the colour development solution (BioRad alkaline

phosphatase substrate kit), all reagents were the same as those used for immunoblot in Example 3. Washes were performed by filling the wells by pipette and emptying them by flicking out over the sink then tapping upside down on paper towels.

After protein coating was completed the wells were washed 2 X with TBS, filled with blocking solution and incubated for 1 hour, then washed 4 X with wash solution. The first well of every row of 12 wells was filled with 200 µL of the appropriate patient serum (diluted 1:32 in antibody buffer) while the next 9 wells received 100 µL of antibody buffer only. 100 µL of the 1:32 dilution was then withdrawn to make serial two-fold dilutions of the patient serum in the next 9 wells. In the first 6 rows, wells 11 and 12 were negative controls with no serum and no protein respectively. In the final 2 rows, wells 11 and 12 were positive controls (receiving a 1:64 serum dilution from a strongly reactive patient). The sera were incubated (covered) for 2 hours, with mixing by pipette after 1 hour. Following 6 X washes with wash solution, each well received 100 µL of secondary antibody solution for 2 hours incubation (covered), with mixing by pipette after 1 hour. Wells were washed 6 X with wash solution, then 2 X with TBS before addition of 100 µL of colour development solution (add 2mL of 5X buffer to 8mL of filtered de-ionised water and dissolve in this two p-Npp tablets just before use) and incubation for 30 mins. The reaction was stopped by addition of 100 µL stop solution (0.4M NaOH) and plates were read at 405nm in a microplate reader. All samples were done in duplicate on the same plate, the mean absorbance readings for each dilution were calculated and the mean absorbance of the blanks was subtracted. Absorbance readings were then multiplied by a standardization factor (the amount required to adjust the positive control to its mean value of 0.280) to allow accurate comparison of samples from different runs. The antibody titre was then determined as the highest dilution giving an absorbance reading greater than 0.100.

Results

Optimisation

Initial optimisation steps showed that a *P. acnes* surface protein concentration of 1.5μg/mL gives a protein coating suitable for ELISA assay of human sera. Two-fold serial serum dilutions from 1:32 to 1:16 384 are suitable for determining antibody titres in most prostate biopsy patients and controls at this concentration of protein coating.

Specificity

The specificity of the assay was confirmed by testing the serum of two rabbits after immunization with the antigen mixture (three doses of 0.5mg antigen given at 21 day intervals). Pre-immunization titres were negative and 1:2 whereas post-immunization titres were 1:32 768 and 1:8192 respectively, confirming that the ELISA can detect serum antibodies against these *P. acnes* surface proteins.

Patient Analysis

Sera from 68 prostate biopsy patients (mean age of 64.5 years, range 46 to 84 years) were analysed by ELISA. The results by ELISA closely reflected the results obtained by immunoblot for individual patients. Patient antibody titres ranged between 1:8 to 1:8192 (median = 1:256), showing that the most strongly reactive patients have anti-*P. acnes* antibody levels around 1000 times higher than the levels found in the most weakly reactive patients. We also analyzed sera from 16 healthy male volunteers (mean age of 55.2 years, range 48 to 70 years) with low serum PSA levels (< 1.5 ng/mL) and no past or present symptoms of prostate disease. These controls had a similar distribution of antibody titres, ranging from 1:64 to 1:8192 (median = 1:256).

Patients were divided into two groups based on anti-*P. acnes* immune reactivity: a high-titre group with antibody titres equal to or greater than 1:1024 (range 1:1024 to 1:8192) and a low-titre group with antibody titres below 1:1024 (range 1:8 to 1:512). Of the 68 patients analysed, 22 (32%) were in the high-titre group and 46 (68%) were

in the low-titre group. The healthy male controls again showed a similar distribution, with six men (37.5%) being in the high-titre group.

Comparison between the high and low-titre patient groups showed that cancer was diagnosed in 13/22 (59%) of high-titre compared to 24/46 (51%) of low-titre patients ($p = 0.62$). No differences were detected when these groups were compared for multiple other parameters commonly associated with prostate disease, including patient age, serum PSA level, prostate volume (a surrogate indicator of benign prostatic hyperplasia), the extent of inflammation and the grade of aggressive inflammation (data not shown).

Separate analysis was then conducted for patients with a diagnosis of prostate cancer (cancer group, Table 1) and patients with only benign tissue detected on biopsy (biopsy-negative group, Table 2). Prostate volume was significantly larger in the biopsy-negative group (median = 51.0 cm^3, range 20.0 to 89.2 cm^3) compared to the cancer group (median = 38.0 cm^3, range 18.8 to 71.3 cm^3, $p = 0.004$). Anti-*P. acnes* antibody titres tended to be higher in the cancer group, which contained all patients with titres of 1:4096 (3 cases) and 1:8192 (1 case) although there was no overall difference (median titre = 1:256 for both cancer and biopsy-negative groups, $p = 0.23$).

Within the cancer group, comparison of high and low-titre patients again did not show significant differences in any of the parameters commonly associated with prostate disease, including the length of cancer (a surrogate for total cancer volume) or the percent of high grade (Gleason Grade 4/5) cancer present in the biopsy cores (Table 1). However in the biopsy-negative group, a trend towards higher PSA levels was apparent in high-titre patients ($p = 0.07$). In particular, high-titre patients were almost 5 times more likely to have a PSA level of 10.0 ng/L or greater ($p = 0.04$) (Table 2).

TABLE 1: Cancer group; comparison of parameters associated with prostate disease in patients with high or low titres of antibodies against *P. acnes*

Cancer Group (n=37)			
	High Titre (n=13) Mean (median) (range)	Low Titre (n=24) Mean (median) (range)	p value
Parameter			
Anti-P. acnes titre	1:2521 (1:2048) (1:1024 - 1:8192)	1:215 (1:256) (1:16 - 1:512)	N/A
Age (yrs)	66.3 (67.0) (55.0 - 79.0)	66.5 (66.5) (46.0 - 84.0)	0.79
Prostate volume (cm^3)	38.4 (35.3) (18.8 - 71.3)	40.8 (38.5) (22.5 - 57.9)	0.40
PSA (ng/mL)	8.6 (5.9) (4.3 - 19.3)	8.7 (7.3) (3.3 - 27.0)	0.69
Cancer length (%)	8.6 (4.9) (0.3 - 34.4)	16.9 (5.4) (0.2 - 98.0)	0.73
% of grade 4/5 cancer	30.0 (10.0) (0.0 - 100.0)	29.0 (20.0) (0.0 - 100.0)	0.77
No. (%) of patients with:			
PSA ≥10.0 ng/mL	4 (30.8)	6 (25.0)	0.72
Inflammation extent (grade 1 or higher)	7 (53.8)	12 (50.0)	1.00
Aggressive inflammation (grade 1 or higher)	4 (30.8)	8 (33.3)	1.00

TABLE 2: Biopsy-negative group; comparison of parameters associated with prostate disease in patients with high or low titres of antibodies against *P. acnes*

Biopsy-negative Group (n=31)			
	High Titre (n=9) Mean (median) (range)	Low Titre (n=22) Mean (median) (range)	p value
Parameter			
Anti-P. acnes titre	1:1479 (1:1024) (1:1024 - 1:2048)	1:178 (1:128) (1:8 - 1:512)	N/A
Age (yrs)	62.4 (65.0) (47.0 - 73.0)	62.2 (62.5) (48.0 - 80.0)	0.85
Prostate volume (cm^3)	55.3 (52.3) (39.1 - 87.3)	48.7 (48.9) (20.0 - 89.2)	0.50
PSA (ng/mL)	10.4 (8.7) (3.9 - 17.4)	6.1 (5.8) (1.3 - 12.0)	0.07
No. (%) of patients with:			
PSA ≥10.0 ng/mL	4 (44.4)	2 (9.1)	**0.04**
Inflammation extent (grade 1 or higher)	7 (77.8)	18 (81.8)	1.00
Aggressive inflammation (grade 1 or higher)	7 (77.8)	13 (59.1)	0.43

Since high antibody titres were associated with high PSA levels in biopsy-negative patients only, we used regression analysis to determine if different factors were influencing the PSA levels in the cancer group compared to the biopsy-negative patient group. In the cancer group, simple linear regression showed that cancer length (R = 0.40) and patient age (R = 0.40) were statistically significant predictors of PSA (Table 3). In multiple linear regression, a stepwise model identified cancer length as the only significant independent predictor of PSA, explaining 13.7% of the total variance in serum PSA in cancer patients (Table 3).

However, in the biopsy-negative patient group, simple linear regression showed that anti-*P. acnes* antibody titre (R = 0.40) was a statistically significant predictor of PSA level while patient age (R = 0.36), prostate volume (R = 0.23) and aggressive inflammation grade (R = 0.20) had lower positive predictive values (Table 4). In multiple linear regression, a stepwise model confirmed anti-*P. acnes* antibody titre as the only significant independent predictor of PSA, explaining 13.2% of the total variance in serum PSA in biopsy-negative patients (Table 4).

The relationship between patient age, prostate volume, aggressive inflammation grade and *P. acnes* antibody titre was further explored with a multiple regression model using the former three variables to predict PSA: adding anti-*P. acnes* titre to this model reduced the predictive values of age by 1.3 times, of prostate volume by 1.4 times and of aggressive inflammation grade by 4.8 times (not shown), to give the final coefficients seen in the block entry model (Table 4). These results suggest that anti-*P. acnes* titre is correlated to serum PSA partly through the mechanisms of increasing age, prostate volume and inflammation, which indicate the development of subclinical benign prostatic hyperplasia (BPH).

TABLE 3: Cancer group; simple and multiple linear regression analysis to determine predictors of serum PSA level

	Standardized Coefficient (R)	Coefficient p value	Adjusted R^2 x 100	Model p value
Simple Linear Regression				
Independent variable				
cancer length (%)	0.40	**0.01**	13.7	**0.01**
Age (yrs)	0.40	**0.02**	13.4	**0.02**
% Grade 4/5 cancer	0.31	0.06	7.30	0.06
Aggressive inflammation (Grade 0-3)	0.04	0.83	0.10	0.83
Prostate volume (cm^3)	0.04	0.82	0.00	0.82
Anti-*P. acnes* titre	0.02	0.92	0.00	0.92
Inflammation extent (Grade 0-3)	0.02	0.93	0.00	0.93
Multiple Linear Regression				
Stepwise model				
cancer length (%)	0.40	**0.01**	13.7	**0.01**

TABLE 4: Biopsy-negative group; simple and multiple linear regression analysis to determine predictors of serum PSA level

	Standardized Coefficient (R)	Coefficient p value	Adjusted R^2 x 100	Model p value
Simple Linear Regression				
Independent variable				
Anti-*P. acnes* titre	0.40	**0.03**	13.2	**0.03**
Age (yrs)	0.36	0.05	12.9	0.05
Prostate volume (cm^3)	0.23	0.20	5.20	0.20
Aggressive inflammation (Grade 0-3)	0.20	0.29	3.90	0.29
Inflammation extent (Grade 0-3)	-0.12	0.42	0.44	0.42
Multiple Linear Regression				
Stepwise model				
Anti-*P. acnes* titre	0.40	**0.03**	13.2	**0.03**
Block Entry Model				
Anti-*P. acnes* titre	0.34	0.08		
Age (yrs)	0.19	0.31		
Prostate volume (cm^3)	0.15	0.42		
Aggressive inflammation (Grade 0-3)	0.01	0.96	9.10	0.18

Conclusions

We have shown that the main factor predicting serum PSA levels in the cancer patient group is the volume of cancer present, whereas the main factor predicting serum PSA levels in the biopsy-negative patient group is their serum titre of antibodies against *P. acnes*.

Previous studies investigating the causes of elevated PSA in biopsy-negative patients without symptoms of prostatitis have identified patient age, prostate volume and the aggressiveness of inflammation as significant predictors, however multivariate analysis consistently identified prostate volume as the predominant predictor of serum PSA [18-20]. Our results also identified patient age, prostate volume and aggressiveness of inflammation as positive predictors of PSA in this patient group, but multiple regression revealed anti-*P. acnes* antibody titre as the predominant independent predictor. The presence of antibody titre in the regression model reduced the predictive values of age 1.3-fold, prostate volume 1.4-fold and aggressive inflammation grade 4.8-fold, indicating that the antibody titre is linked to PSA levels partly through these factors. Since age-associated increases in both prostate volume and serum PSA mainly reflect subclinical BPH [21] which is associated with marked inflammation [22], our results suggest that *P. acnes* infection of the prostate gland may be related to development of BPH through generation of inflammation. This is in accord with our finding of significantly larger prostate volumes in the biopsy-negative patient group, indicating the presence of subclinical BPH.

Although the volume of cancer was the main factor influencing PSA levels in the cancer group, we did note a tendency for higher anti-*P. acnes* antibody titres in the cancer patients compared to the biopsy-negative patients. Certainly all patients with titres of 1:4096 (3 cases) and 1:8192 (1 case) were in the cancer group. It is possible that screening of a larger patient group may confirm a significant association between high anti-*P. acnes* antibody titres and development of prostate cancer.

While our ELISA assay has shown some evidence of relationships between high anti-*P. acnes* antibody titres and inflammation-related prostate diseases such as BPH and prostate cancer, the assay is clearly not specific for detecting *P. acnes* infection of the prostate gland. High antibody titres were also found in some subjects without infection of the prostate gland (for example in female controls who do not have a prostate gland) and in some healthy male controls with very low PSA levels, who are unlikely to have infection of the prostate gland. In these cases, high immune reactivity to *P. acnes* may

reflect infection in other regions of the body known to be inhabited by *P. acnes* , for example in the urinary tract, intestinal tract, genital tract or on the skin. Alternately they may indicate individuals with a hypersensitive immune response to normal levels of *P. acnes* antigen exposure. These sensitized individuals may be likely to develop a strong inflammatory reaction in response to infections involving *P. acnes*, including infection of the prostate gland, thus may be at increased risk of developing inflammation-related prostate diseases such as BPH and/or prostate cancer.

Serum PSA level is currently the main diagnostic test used to screen for prostate cancer, but it has low specificity for detecting prostate cancer because numerous other factors can also cause raised serum PSA levels, including BPH and prostatitis (prostatic infection and/or inflammation)[16,17]. We propose that ELISA testing for immune reactivity to *P. acnes* may be a useful supplement to PSA screening and prostate biopsy, capable of identifying patients who either have *P. acnes* infection of the prostate gland or are hypersensitve to *P. acnes* antigens, and who therefore have increased risk of developing prostate diseases including cancer even if their biopsy results are currently negative for cancer. This may be particularly relevant for patients with both high PSA levels and high *P. acnes* titres, but with biopsy results negative for cancer. These patients could then be given treatment with antibacterial agents such as antibiotics which may prevent the development of prostate disease including cancer.

EXAMPLE 5

Assessment of local immune reaction to *P. acnes* surface/secreted proteins using IgA ELISA to test urine, prostatic secretions or ejaculate.

ELISA testing of serum to detect antibodies against *P. acnes* can identify individuals with raised antibody titres, suggesting the presence of a current or recent *P. acnes* infection. However, this method does not specifically identify men with *P. acnes* infection of the prostate gland. ELISA testing of samples such as urine, expressed prostatic secretions or ejaculate can be conducted using standard secondary antibodies that detect human IgG, however these methods are also not specific to urinary tract or

prostate gland infection because a large proportion of the IgG antibodies in bodily fluids such as urine is derived from the serum [23].

In contrast, the serum contains 5-fold lower levels of IgA antibodies compared to IgG [24]. IgA is locally produced within mucosal lymphoid tissues [25], thus IgA levels in bodily fluids may be more representative of local infections. ELISA to detect coliform-specific IgA in urine has been used to assess the role of these bacteria in urinary tract infections [26]. Similarly ELISA to detect *Chlamydia trachomatis*-specific IgA in ejaculate has been used to assess the role of these bacteria in prostatic infection [27]. Development of an ELISA that is more highly specific for infection in a particular organ may be possible using secondary antibodies that detect secretory IgA, which comprises only 1.6% of the total IgA in serum but is the predominant antibody produced by local mucosal tissue [24]. Secretory IgA is comprised of IgA polymers attached to secretory component and is formed during transport of IgA through the epithelial cells lining mucosal surfaces and out into the lumen of the mucosa [25]. ELISA to detect coliform-specific secretory IgA in urine has been used to assess the role of these bacteria in urinary tract infections [26], while detection of total secretory IgA in prostatic secretions and prostate tissue by radioimmunoassay has been used to assess the role of this antibody in prostatic infections [28].

Methods

ELISAs to detect IgA and secretory IgA are carried out as described in Example 4, with the following exceptions:

1. Instead of patient serum, the primary antibody solution contains either urine, expressed prostatic secretions, or ejaculate.
2. Instead of anti-human IgG, the secondary antibody used is anti-human IgA (for detection of IgA) or anti-human secretory component (for detection of secretory IgA).

Overall Conclusions

In patients undergoing prostate biopsy due to either elevated serum PSA level or abnormal DRE examination, there is a significant positive association between high PSA level (≥ 8.0 ng/mL) and high *P. acnes*-specific antibody titre (≥ 1:1024). However the prostate biopsy patients with high *P. acnes*-specific antibody titres (group 1) did not have significantly higher values for any of the traditional parameters known to contribute to raised PSA levels, including age, prostate volume, presence of cancer in the biopsy specimen, amount of cancer in the biopsy specimen or grade of cancer in the biopsy specimen.

Since raised PSA levels can be caused by prostatic infection and inflammation [18-20] it appears likely that infection of the prostate with *P. acnes* may have caused both the raised PSA levels and the high degree of *P. acnes*-specific immune reactivity in this subset of patients (group 1).

High levels of *P. acnes*-specific antibodies are clearly not specific to patients with prostate disease, since they can also be found in subjects without infection of the prostate gland (for example in female controls who do not have a prostate gland). In these cases, high immune reactivity to *P. acnes* may reflect infection in other regions of the body known to be inhabited by *P. acnes* , for example in the urinary tract, intestinal tract, genital tract or on the skin.

Serum PSA level is currently the main diagnostic test used to screen for prostate cancer, but it has low specificity for detecting prostate cancer because numerous other factors can also cause raised serum PSA levels, including benign prostatic hyperplasia and prostatitis (prostatic infection and/or inflammation)[19]. We propose that testing for immune reactivity to *P. acnes* may be a useful supplement for PSA screening and prostate biopsy, capable of identifying patients with *P. acnes* infection of the prostate gland and therefore with increased risk of developing prostate diseases including cancer even if the biopsy results are currently negative for cancer. This may be particularly

relevant for patients with both high PSA levels and high *P. acnes* titres (as in our patient group 1), but with biopsy results negative for clinically significant cancer. These patients could then be given treatment with antibacterial agents such as antibiotics which may prevent the development of prostate disease including cancer.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

All publications mentioned in this specification are herein incorporated by reference. Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia or elsewhere before the priority date of each claim of this application.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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CLAIMS

1. A method for diagnosing prostatic disease and/or risk of prostatic disease in men comprising obtaining a body fluid sample from a patient and assaying for the presence of antibodies specific for *P. acnes* in the body fluid sample.

2. The method according to claim 1 wherein the prostatic disease is selected from the group consisting of prostate cancer, prostatitis and benign prostatic hyperplasia.

3. The method according to claim 1 or claim 2 wherein the body fluid sample is selected from the group consisting of serum, plasma, urine, prostatic secretions and ejaculate.

4. The method according to any one of claims 1 to 3 wherein the method comprises assaying for the presence of antibodies specific for *P. acnes* secreted proteins or membrane-anchored surface proteins or both.

5. The method according to claim 4 wherein the secreted proteins and/or membrane-anchored surface proteins include proteins from *P. acnes* types IB and II.

6. The method according to any one of claims 1 to 5 wherein the antibodies are IgA.

7. The method according to any one of claims 1 to 6 wherein the antibodies are secretory IgA.

8. The method according to any one of claims 1 to 7 wherein the method comprises quantitating the antibody titre of the body fluid sample to *P. acnes* protein.

9. The method according to any one of claims 1 to 8 further comprising the step of measuring the level of prostate specific antigen present.

FIGURE 1

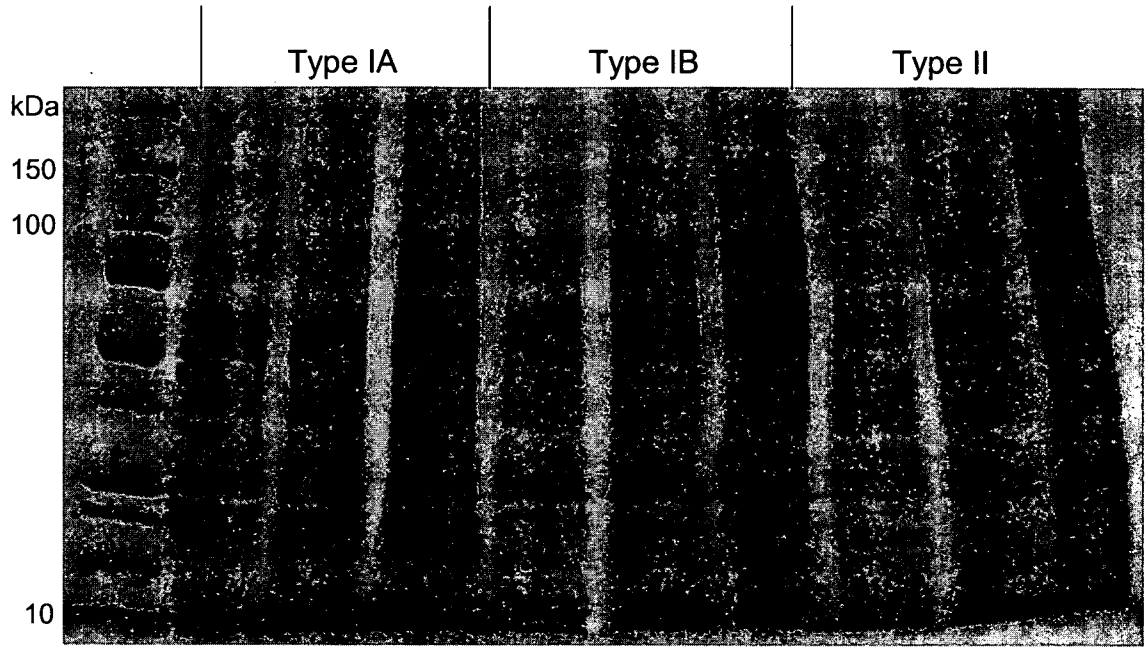


FIGURE 2

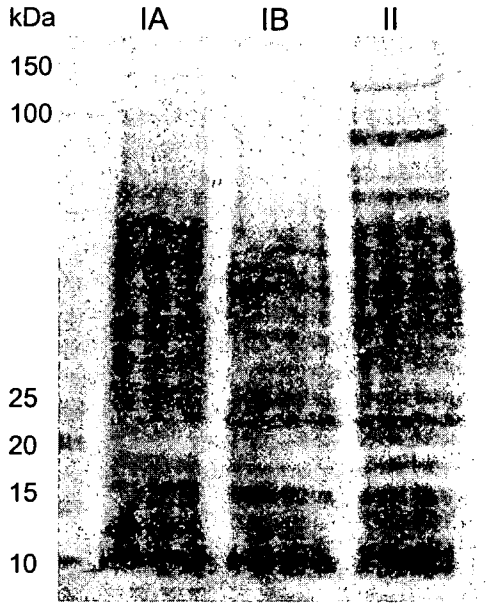
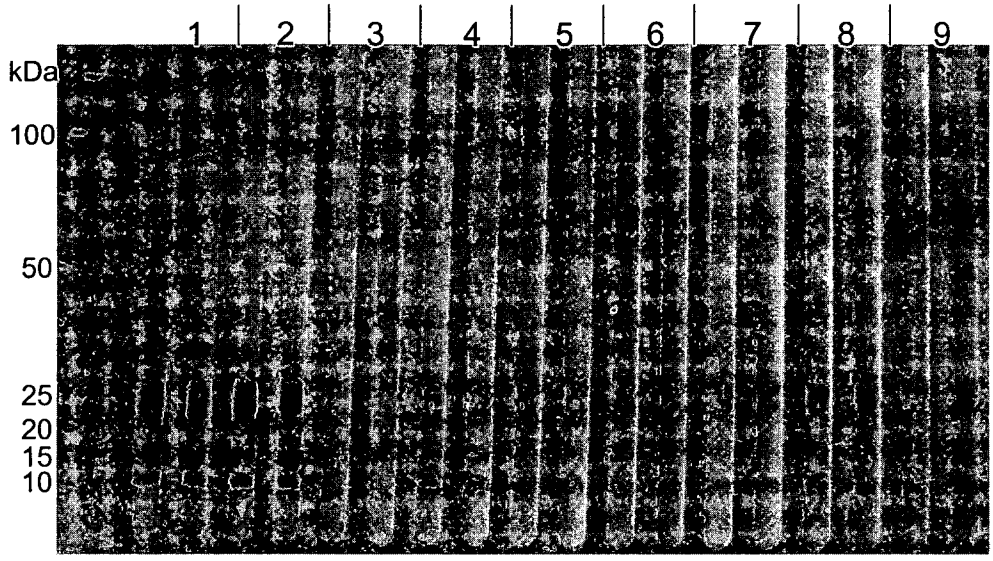


FIGURE 3



INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2008/000105

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C12Q 1/04 *G01N 33/68* (2006.01) *G01N 33/50* (2006.01) *G01N 33/53* (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q/IC or G01N/IC

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN: WPIDS, JAPIO, CAPLUS, MEDLINE, BIOSIS. Key words: bacteria# or acnes or propionibact? or (propioni (w) bacter?) or (P (w) acnes); prostat?, antibody? Or immunoglobulin?, protein? or (membrane (w) anchore?)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SHANNON, BA et al., "The antibody response to *Propionibacterium acnes* is an independent predictor of serum prostate-specific antigen levels in biopsy-negative men", BJU International (10th Sep 2007 Epublication), Vol 101(4): 429-435. See whole document	1-9
A	WO 2005/087929 A1 (TISSUGEN PTY LTD), 22 September 2005 See whole document	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
20 February 2008	27 FEB 2008
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **HEMA INDRASAMY** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : (02) 6283 3179

INTERNATIONAL SEARCH REPORT

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	COHEN RJ, et al., "*PROPIONIBACTERIUM ACNES* ASSOCIATED WITH INFLAMMATION IN RADICAL PROSTATECTOMY SPECIMENS: A POSSIBLE LINK TO CANCER EVOLUTION?", The Journal of Urology (2005), Vol 173 (6): 1969-1974. See whole document	
A	SHANNON BA, et al., "Polymerase chain reaction-based identification of *Propionibacterium acnes* types isolated from the male urinary tract: evaluation of adolescents, normal adults and men with prostatic pathology", BJU International (2006), Vol 98(2): 388-392. See whole document	
A	SHANNON BA, et al., "Links between *Propionibacterium acnes* and prostate cancer, Future Oncology (2006), Vol 2(2): 225-232. See whole document	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2008/000105

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	2005087929	AU	2005221729	CA	2559390	EP	1725662

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX