Cross–reactivity of antibodies against microbial proteins to human tissues as basis of Crohn's disease and Sjogren's syndrome

Peilin Zhang [1*]., Lawrence M. Minardi [1]., J. Todd Kuenstner [1]., Stephen M. Zekan[1].,

Feng Zhu [2]., Yinling Hu [2], Rusty Kruzelock. [3]

[1] PZM Diagnostics, LLC, Charleston, WV 25301

[2] Center for Cancer Research, National Cancer Institute, Frederick, MD 21702

[3] West Virginia Regional Technology Park, South Charleston, WV 25309

[*] Correspondence:

Peilin Zhang, MD., Ph.D.
PZM Diagnostics, LLC
500 Donnally St., Suite 303
Charleston, WV 25301
Tel: 3044447505
Email: pzmllc@gmail.com

Abstract

Background

Autoimmune disease is a systemic inflammatory response with autoantibodies. We investigated anti-microbial antibodies in Crohn's disease (CD), and Sjogren's syndrome (SS) and their roles in pathogenesis of these autoimmune diseases.

Methods

Western blot was used to determine the reactivity of the plasmas from patients with CD and SS as primary antibodies against the whole microbial extracts. The microbial proteins reactive to patients' plasmas were identified and the modified ELISA assays were used to determine the blood levels of antibodies against these microbial proteins in patients with CD and SS. Antibodies against the microbial proteins are used for immunohistochemical staining of normal human tissue.

Interpretation

A group of 7 microbial proteins were identified reactive to the plasmas of patients with CD and SS including DNA-directed RNA polymerase B (RPOB), and elongation factor G (EF-G) from *Staphylococcus aureus* and *Staphylococcus pseudintermedius* (*S. aureus* and *S. pseudintermedius*), ATP synthase alpha and heat shock protein 65 (*Hsp65*) from *Mycobacterium avium subspecies paratuberculosis* (MAP), elongation factor Tu (EF-Tu) and outer membrane porin C (ompC) from *Escherichia Coli* (*E. coli*). One unknown protein from *S. aureus* is also identified. Anti-microbial antibodies can cross-react with human tissues. The antibody levels against these microbial proteins are significantly elevated in the patients with CD and SS. The presence of anti-microbial antibodies indicates the failure of the innate immunity and the cross-reactivity of the anti-microbial antibodies to human tissue provides a new mechanism of pathogenesis of autoimmune diseases such as CD and SS.

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Autoimmune diseases are a spectrum of clinical conditions characterized by systemic manifestation with circulating antibodies against the patient's own tissues with the exception of CD in which no identifiable autoantibody is known[1]. Genetic susceptibility is an essential pre-requisite condition for the pathogenesis of autoimmune diseases, although the molecular mechanisms of most autoimmune diseases are unclear [1]. Recent advances of microbiome research demonstrated that the surface area of human body including body cavities is covered by a plethora of microbes (commensal), and these microbes are essential in host defense against pathogens, innate and adaptive immunity development and prevention of a variety of diseases [2,3]. The symbiotic relationship of the human body with its own microbes becomes an interesting issue in the pathogenesis of infection, immunity and various degenerative diseases.

Decades ago the discovery of anti-streptolysin O antibody in the circulation of patients with streptococcal infection (pharyngitis) cross reacting with human valvular tissue and renal glomerular tissue leading to valvular heart disease and glomerulonephritis provides the basis of understanding the mechanism of streptococcal infection and the related disease process, which led to new treatment and preventive strategy [4,5]. In our effort to determine the role of mycobacterial infection in CD, we found there are elevated antibody levels in the patient's circulation against a panel of microbial proteins. These anti-microbial antibodies can cross-react with human tissue, potentially leading to dysfunction of human tissue and various clinical conditions. The elevated levels of anti-microbial antibodies can be used as diagnostic tools for assessment of the relationship and compatibility between the host and its own microbes, and the presence of anti-microbial antibodies provide a new direction of research in understanding the mechanism of autoimmune diseases.

Methods

1, Western blot analysis using the microbial cell extracts and the patients' plasmas as primary antibodies:

In order to determine if the human plasma from the patients with CD and SS react to the microbial proteins, the whole microbial extracts from *S. aureus, S. pseudintermedius, E.*

coli, *MAP* and *MAH* are prepared and Western blot analysis is performed as described [6]. Briefly, the microbial culture (1.0 ml) in the appropriate media is centrifuged at 15,000 g for 5 minutes. The microbial cell pellet is washed with 100% acetone once, and resuspended in 1% SDS at 4^0C overnight to extract cellular proteins. The protein concentration is determined by using Qubit protein quantification kit (Fisher Scientific). The whole microbial extracts (20 ug) are subjected to 10% SDS-polyacrylamide gel electrophoresis and the cellular proteins are electroblotted onto nitrocellulose membrane. The microbial proteins on the nitrocellulose membrane are incubated with the plasma (1 to 50 dilution) from patients with CD and SS at room temperature for 2 hours (or 4^0C overnight), and secondary antibodies and detection systems are provided by using the Pierce enhanced chemiluminescent detection kit (Fisher Scientific) according to the manufacturer's instruction.

2, Identification of the microbial proteins by immunoprecipitation and mass spectrometry:

Seven microbial proteins showed in Figure 1 are precipitated by using the same patient's plasmas as primary antibodies. Briefly, the bacterial/mycobacterial cell extracts (50 ug/200ul) were incubated with 50 ul plasma from the patients and 50 ul protein A-coupled agarose beads (Fisher Scientific) at 4^0C overnight and the specific microbial proteins binding to the plasmas are captured by using the protein A-agarose column (Fisher Scientific). The captured microbial proteins are analyzed by Nanospray liquid chromatography/mass spectrometry analysis (LC/MS/MS) with database search by Poochon Scientific, LLC (Frederick, MD) through contract work. A list of microbial proteins are obtained, and only the proteins of similar molecular weights are of interest for further validation test (Table 1).

3, Immunohistochemical staining of human tissue with specific anti-microbial antibodies:

Frozen section slides of human tissues were purchased commercially and used directly for immunohistochemical staining [6]. The primary antibodies against EF-Tu of

Acinetobacter, RPOB from *E.coli* and *hsp65* of *Mycobacterium tuberculosis* are diluted at 1:200, and applied to the frozen section tissue slides. The primary antibodies are incubated with the tissue slides for 1 hour at room temperature. The slides are washed 3 times and the secondary antibodies against the rabbit or the mouse IgG conjugated with horseradish peroxidase (HRP) are applied to the tissue slides. The signals are developed by using DAB enhanced detection system kit from Pierce (Fisher Scientific).

4, Modified Sandwich enzyme-linked immunosorbent assay (ELISA assay):

Once the microbial protein identities are known, commercial antibodies are sought, and there are commercial antibodies against various proteins of different species. Depending upon the homology between the amino acid sequences, cross-species antibody may or may not bind to the same protein of another species. Based on the homology of amino acid sequences to human proteins shown in Table 1, a list of commercial antibodies are purchased (Table 1), and tested in the modified sandwich ELISA assays [6]. Briefly, the 96-well plate (ELISA plate) is coated with the specific antibody in Table 1 in coating buffer (at the concentration of 1.0 ug/ml) at 4^0C overnight [6]. The microbial whole cell extracts were added to the wells at the concentration of 20 ug/ml at room temperature for 2 hours. After washing, the whole plasma of 50 ul was added from the patients with CD and SS as well as the normal healthy controls. The plasma was incubated in the plate at room temperature for 1 hour, and the plate was washed with PBST. The signals are developed by using anti-human IgG conjugated with horseradish peroxidase HRP/TMB/acid stop buffer and the absorbance reading at 450 nm by using Versamax microplate reader from Molecular Device Inc.

The plasma samples from the patients with Crohn's disease and Sjogren's syndrome were collected from various parts of the world through the commercial testing service at PZM Diagnostics (Charleston, WV, www.crohnsmanagement.com) with patients' consent requisition forms on file. The healthy controls were obtained from the local clinics with the patients' consent.

Findings

1, Identification of microbial proteins reactive to human plasma of Crohn's patients: During our initial testing for MAP from the blood of patients with CD, we found a significant percentage of patients positive for the presence of antibody within the blood against MAP whole cell extract (over 70%, data not shown). We wanted to determine which specific antigen(s) from the MAP whole cell extract are reactive to patients' plasmas. We performed Western blot analysis using the MAP whole cell extract (20 ug) on the SDS polyacrylamide gel electrophoresis, and patients' plasmas as a primary antibodies (1:50 dilution). We found that there is one protein from MAP and MAH strongly reactive to patient's plasma (Figure 1A, M60). There are multiple bacterial proteins from *S. aureus* and *S. pseudintermedius* reactive to the plasma of the same patient (S75, S55, and S27). There are patients only reactive to proteins from *S. aureus* or *Escherichia. Coli* but not both (Figure 1B and 1C, S135, E42, E39).

We used the same patients' plasmas and the bacterial extracts to perform the immunoprecipitation and mass spectrometry to determine the identities of these bacterial/mycobacterial proteins reactive to patients' plasmas. Nanospray LC mass Spectrometry and protein identification were performed at the Poochon Scientific LLC, Frederick, MD, and a list of potential bacterial/mycobacterial candidates are provided to us after the database search, and only the proteins with same molecular weights are considered for further validation (Table 1). There are seven microbial proteins identified from the four microbes: S135 is determined to be DNA-directed RNA polymerase subunit B (RPOB) from *S. aureus.* RPOB is a critical enzyme for bacterial gene transcription. It is also a clinically relevant target for rifampin, one of the most important drugs for tuberculosis. Mutation of the RPOB gene confers rifampin resistance in *E.coli, S. aureus* and *Mycobacterium tuberculosis*. S75 represents a bacterial protein from *S. pseudintermedius*, a commensal bacteria on the surface of a domestic dog with potential to be pathogenic in humans [7,8]. The strain of *S. pseudintermedius* was isolated from a dog with a skin ulcer (hot spot, moist dermatitis), and the isolate was confirmed by partial 16S rDNA and whole genomic sequencing (data not shown). Using Western blot analysis and immunoprecipitation of plasmas of two separate patients with CD as the primary antibodies and using mass spectrometry, S75 was determined to be elongation factor G

(EF-G) from *S. aureus*. EF-G is a critical regulator of bacterial protein synthesis, and it is present in all bacteria species. The human homologue is present in the mitochondria (G elongation factor mitochondrial 1, GFM1), and it plays similar roles in human protein biosynthesis. The homology between the bacterial protein and the human protein at the amino acid levels is 61% (Table 1). M60 is a protein from MAP/MAH, and M60 is determined to be heat shock protein 65 (*hsp65*). The human homologue is the human *hsp60*, a molecular chaperone known to be an autoantigen in cancer patients and other autoimmune diseases [9,10]. S55 is a bacterial protein seen in *S. aureus* and *S. pseudintermedius* and it is determined to be ATP synthase alpha from *S. aureus*, and *M. tuberculosis*. The human homologue is present in the mitochondria (ATP5a), and it plays an important role in ATP generation and energy biosynthesis. E42 is a bacterial protein from *E. coli* identified by Western blot using a plasma from a patient with mixed CD and SS. Further identification showed the bacterial protein is consistent with elongation factor Tu (EF-Tu) from *E.coli*. EF-Tu is one of the most abundant proteins from *E.coli*, and there is a human homologue of the bacterial EF-Tu from *E.coli*, EF-Tu mitochondrial precursor (TUFM). There is no human disease associated with this protein up to date. E39 is a protein from *E.coli* reactive to plasma of the same patient with mixed CD and SS by Western blot analysis. The protein is identified as outer membrane porin C (ompC) from *E. coli*. The bacterial protein omp C from *E.coli* has been previously used in the Prometheus inflammatory bowel disease (IBD) panel (San Diego, CA) [11]. There is no human homologue of ompC from *E.coli*, and it is reported that a significant percentage of CD patients (up to 40%) showed anti-ompC antibody in their circulation [12]. S27 is a protein from the *S. pseudintermedius* strongly reactive to the plasma of a young female patient with CD and the protein is determined to be the uncharacterized lipoprotein SACOL0083 (Genbank accession #Q5HJR9.1). There is no previous documentation of this protein.

2, Specific anti-microbial antibodies cross-react to normal human tissues:

To determine if the specific antibodies against the microbial proteins can react to human tissue, and the localization of the target antigens in human tissue, immunohistochemical staining techniques are employed. Specific antibodies from various sources were

obtained commercially (Table 1, www.linscottsdirectory.com). Specific polyclonal antibody against EF-Tu from Acinetobacter (bacteria) raised in rabbit can directly interact with human thyroid tissue with strong nuclear signal (Figure 2 A-C, A-negative control, B and C at 100X and 200 X magnification). Interestingly, the human homologue of the EF-Tu is a mitochondrial precursor, and TUFM (human) is located within the mitochondria (cytoplasmic fraction), instead of nuclei. The significance of this finding is unclear. Specific monoclonal antibody against *hsp65* from *M. tuberculosis* can also directly interact with normal thyroid tissue (Figure 2, D-F, D-negative control, E and F at 100 X magnification), and the reactivity signals are seen within the cytoplasm of the normal thyroid follicular cells. Polyclonal RPOB antibody only react to the skeletal muscle in a specific manner in which the signals are fine granules or dots in the cytoplasmic membrane (Figure 2, G-I, G-negative control, H and I at 100 X magnification). No nuclear signals are seen. None of the antibodies are reactive to the salivary gland tissue or blood nucleated cells.

3, Elevated levels of anti-microbial antibodies in patients with CD and SS:

The specific polyclonal and monoclonal antibodies against the microbial proteins (Table 1) are used for modified sandwich ELISA assays to determine the anti-microbial antibodies in the blood of the patients with CD and SS. In total, 44 plasma samples are available from the CD patients with 31 normal healthy controls, and 26 plasma samples from SS patients (Figure 3). In the CD patients, 41 patients' plasma samples were positive for one or more markers (93%; all markers were positive in 14 patients, 4 markers were positive in 7 patients, 3 markers were positive in 8 patients, 2 markers were positive in 8 patients, 1 marker was positive in 4 patients, all markers were negative in 3), 14 patients were positive for all five markers (32%), and 3 patients were negative for all the five markers (7%). In contrast, there are 6 patients positive for one or more markers in 31 controls (19%) (All markers were positive in 0 patient, one or more markers were positive in 6 patients). The levels of all five markers are significantly elevated in CD patients compared to the normal controls by unpaired Student *t-test* ($p=0.031$ for *hsp65* to $p<0.0001$ RPOB and EF-Tu) (Figure 3A).

Among the five serological markers, *hsp65* from *Mycobacterium* appears less differentiating with overlapping mean data points, and it is difficult to separate the patients versus the normal controls with large overlapping results and the borderline range, although the difference of the levels in patients and the controls is significant by the unpaired Student *t-test* (p=0.031). All other four markers are easily separable between the CD patients and the normal controls with small overlapping ranges (Figure 3A). There is a cutoff value in each marker when it is measured, and each cutoff value is determined separately based on the property of each antibody used (Figure 3A). We did not correlate the disease severity with specific antibody titers, and we did not test if these serologic markers are useful for monitoring the treatment effect or efficacy.

In total, 26 plasmas from patients with SS are available for analysis, and 22 patients are positive for one or more markers (83%; 6 patients were positive for all markers, 4 patients were positive for 4 markers, 7 patients were positive for 3 markers, 4 patients were positive for 2 markers, 1 was positive for 1 markers), and 4 patients are negative for all markers (17%) (Figure 3B). All five markers showed statistically significant differences in the SS patients versus the controls by the unpaired Student *t-tests* (Figure 3B).

Interpretation

The patient population of all autoimmune diseases is increasing and the underlying causes of all these autoimmune diseases are unknown. Much of the research effort is directed to discover the autoantibodies, and the treatment strategy is directed to suppress the immune system through various mechanisms. We have found a group of antibodies against the microbial proteins and these microbes commonly cover the surface of the human body or in the case of mycobacteria, are widely present in the environment such as water and soil. *S. pseudintermedius* is a commensal bacterium on the surface of domestic dogs, and there are millions of families with domestic pets in the US.

There is a symbiotic relationship between the common microbes and the human hosts. In classic immunology, the pathogenic microbes are cleared by the innate immunity system (non-specific immunity). The development of specific circulating antibody against

specific microbial protein is likely a result of the failed innate immunity which consists of non-specific defense system such as body barriers including surface skin and mucosa lining through body cavities, and the blood defense system including neutrophils, macrophages, mast cells, eosinophils, and complement. The failure of clearance of the microbes from the circulation by the innate immune system network may directly reflect the genetic susceptibility to CD[13], SS and other autoimmune diseases. It also indicates an invasion or penetration of the skin/gut mucosal barrier into the blood circulation, leading to transient or persistent bacteremia.

We have discovered 7 proteins from 4 different types of microbes, *S. aureus, S. pseudintermedius, MAP*, and *E. coli.* *S. aureus* and *E. coli* are the most abundant bacteria on the body surface and in the gut. S. *pseudintermedius* is one of the most abundant bacteria in domestic dogs. *MAP* is present in the environment such as soil, water and dairy products. These microbial proteins are also highly homologous to the human homologues, and the antibodies against the microbial proteins can directly interact with the human tissues. Our immunohistochemical staining results demonstrated that the specific monoclonal or polyclonal antibodies raised against the microbial proteins can react to the human tissues. It is also important to note that there is high homology of the amino acid sequences of these microbial proteins between the microbes such as *S. aureus* and *E. coli*, and the antibodies against *S. aureus* can also react to the same protein antigen from the *E. coli*. Cross-reactivity or molecular mimicry appears to be the key in understanding the mechanism of the underlying diseases caused by these common microbes. Furthermore, the microbial proteins identified above are critical for survival of the microbes except for the unknown lipoprotein (S27) from the *S. aureus*. The elongation factors (EF-G and EF-Tu) are parts of the bacterial ribosomes targeted by some antibiotic families, and the studies to understand the relationship of the microbes and anti-microbial antibodies with antibiotic usage and mitochondrial function will be important.

In a clinical setting, testing anti-microbial antibodies can potentially be used for confirmation of clinical diagnosis. The anti-microbial antibody levels will likely reflect the failure of the innate immune system, or transient/persistent bacteremia, or both. The

presence of the specific antibodies against the microbial proteins within the circulation of a patient suggests an entirely different mechanism of pathogenesis of autoimmune diseases. Ultimately, autoimmune diseases are likely triggered by the infectious agents in genetically susceptible individual, and the infectious agents are likely the common microbes on the surface of human body, or in the gut or in the environment. The discovery of the antibodies against the microbial proteins in CD and SS may provide a new direction of research for understanding the autoimmune diseases, leading to new therapeutic strategy for a large population of patients with autoimmune diseases.

Figure legend:

Figure 1: Identification of microbial proteins using Western blot analysis and the whole plasma of patients with CD. Various microbial cell extracts are subjected to SDS-PAGE analysis. The protein extracts on the membranes were incubated with whole plasma with 1 to 50 dilutions from the patients with CD and/or SS. Three patients' plasmas, A, B and C were used for analysis.

Table 1: List of the microbial proteins identified by Western blot analysis, immunoprecipitation and mass spectrometry and the commercial antibodies. The same patient's plasma was used as for the Western blot analysis in Figure 1 for immunoprecipitation. Mass spectrometry study was performed by Poochon Scientific LLC, Frederick, MD.

Figure 2: Cross-reactivity by immunohistochemical staining of the thyroid tissue and skeletal muscle. A-C: Polyclonal anti-EF-Tu antibody specific to Acinetobacter baumannii and thyroid tissue. A: negative control with no added antibody against EF-Tu at 100 X magnification with hematoxylin (blue); B: positive signals (brown, HRP with DAB system) are seen in the nuclei of the thyroid follicular cells at 100X magnification; C: positive signals are present in the nuclei of the thyroid cells at 200X magnification. D-E: Cross-reactivity of specific monoclonal antibody against hsp65 from *M. tuberculosis* with the normal thyroid tissue. The signal is in the cytoplasm of normal thyroid follicular cells. D: negative control, E and F: staining signals within the cytoplasm of the normal thyroid tissue at 100 X and 200X magnifications. G-I: Cross-reactivity of specific polyclonal antibody against RPOB form *E.coli* with the skeletal muscle. The reactivity signals are fine-granules and dots within the cytoplasm. G: negative control without antibody, H and I showed reactive signals within the cytoplasm and membranes in skeletal muscle at 100X and 200 X magnification.

Figure 3: Blood levels of antibodies against the microbial proteins within the plasmas from the patients of CD, SS and the healthy controls by modified ELISA assays. The whole plasmas from the patients with CD and SS were used for the assays. The p-values

were determined by the unpaired Student t-test. A: Blood levels of antibodies from CD patients; B: Blood levels of antibodies in SS patients.

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PZM Diagnostics LLC is a privately owned specialty microbiology lab registered in the State of West Virginia. There is no financial conflict of interest.

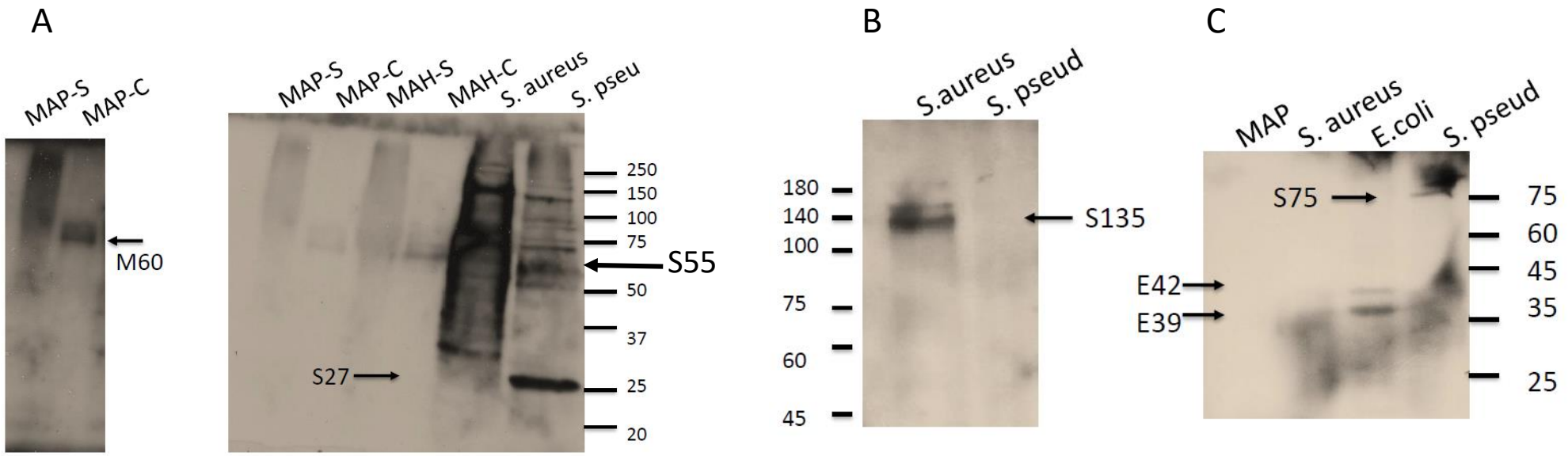

Figure 1 : Western blot analysis of various microbial proteins using the plasmas of the patients with Crohn's disease. Panel A, B and C were using the plasmas from patients A, B and C. MAP-S, MAP culture supernatant, MAP-C, MAP culture cell pellet, MAH-S, MAH culture supernatant, MAH-C, MAH culture cell pellet, S pseud-S pseudintermedius.

Table 1. Protein Identities by mass spectrometry and antibody cross-reactivity

ID	Full name	Homology to human		Immunogen	Reactive to human
		Identities	Positives		
S135	--- DNA-directed RNA polymerase subunit B from *S. aureus* (RPOB)	30%	47%	E. coli	Yes
S75	--- Elongation factor G from *S. pseudintemedius* (EF-G)	42%	61%	Human	Yes
M65	-- Heat shock protein 65 from *M. paratuberculosis* (HSP65)	47%	68%	M. tuberculosis	Yes
S55	--- ATP synthase subunit alpha from *S. pseudintermedius* (ATP5a)	60%	74%	Human	Yes
E42	--- Elongation factor Tu from *Escherichia Coli* (EF-Tu)	56%	75%	Acinetobacter	Yes
E39	--- Outer membrane porin protein C from *Escherichia Coli* (ompC)	No human homologue		Not tested	
S27	--- Uncharacterized lipoprotein SACOL0083 (uncharacterized protein)			Not tested	

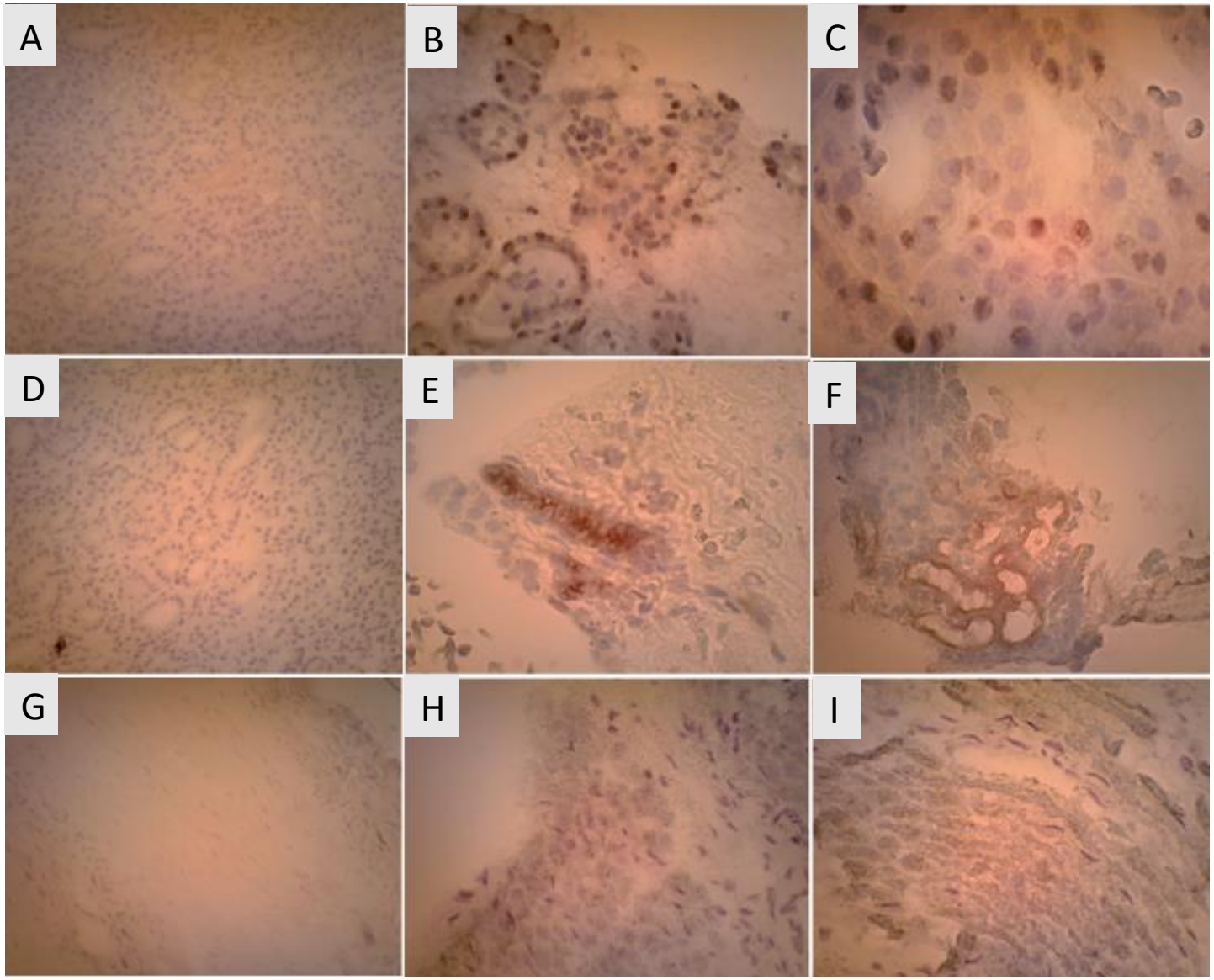

Figure 2: Cross-reactivity of anti-microbial antibodies against human tissue. A-C: IHC stains of human thyroid tissue using polyclonal antibody against EF-Tu from Acinetobacter. D-F: Normal thyroid tissue with monoclonal hsp65 antibody of M. tuberculosis. G-I: normal skeletal muscle with polyclonal anti-RPOB antibody of E. coli.

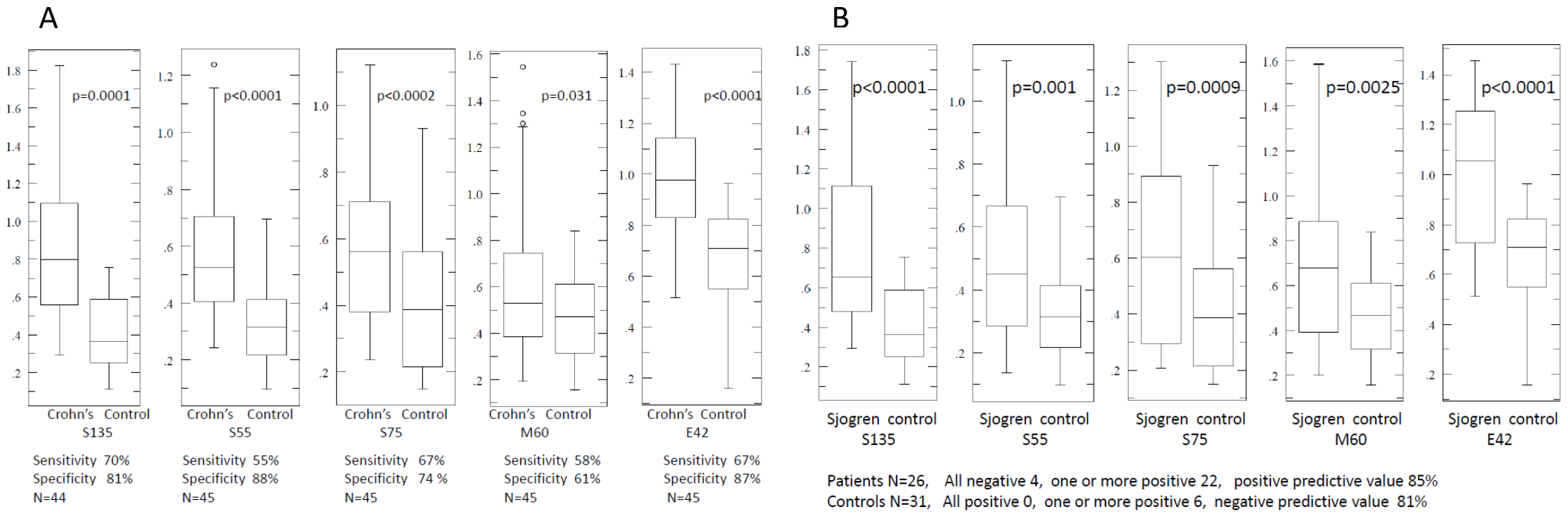

Figure 3: Blood levels of anti-microbial antibodies in CD and SS. A: The blood levels of anti-microbial antibodies in CD and controls, B: The blood levels of the antimicrobial antibodies in SS. The y-axis is the raw OD450 reading. The data is presented as boxplot by using Student *t-test*.