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Graduate Studies

DEVELOPMENT OF A SERUM-ANTIBODY TEST SPECIFIC FOR A BACTERIUM ASSOCIATED WITH RHEUMATOID ARTHRITIS PATIENTS

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Clinical Microbiology concentration

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DEVELOPMENT OF A SERUM-ANTIBODY TEST SPECIFIC FOR A BACTERIUM ASSOCIATED WITH RHEUMATOID ARTHRITIS PATIENTS

By Andrew LeSage

We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science—Clinical Microbiology.

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ABSTRACT

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Rheumatoid arthritis (RA) is an incurable inflammatory autoimmune disease of synovial joints that affects nearly 1% of the global population and an estimated 1.36 million Americans. The disease has significant economic impacts—resulting in annual healthcare expenses totaling $19 billion annually. Pathogenesis is driven by a complex interplay between genetic and environmental factors. Recent literature suggests the presence of microbial communities in synovial joints of both RA patients and normal healthy donors (NHDs). Further analysis revealed that the Raoutella genus of bacteria was present in the synovium of RA patients, but not NHDs. This is suggestive of a possible involvement of the synovial microbiome in the pathogenesis of RA. In this project, pure culture of Raoutella ornitholytica and R. planticola were established and used to generate bacterial whole cell lysates. Each lysate was screened against 10 RA patient and 5 normal healthy donor human serum samples. Checkerboard titrations were performed to optimize the assay and the indirect ELISA protocol established in this study will be used in future lab projects.
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INTRODUCTION

Rheumatoid arthritis (RA) is an incurable inflammatory autoimmune disease of the joints that affects nearly 1% of the global population. An estimated 1.36 million Americans are living with the condition and the number of cases is steadily increasing as the general population ages (1, 2). Once diagnosed, most patients require ongoing treatment and medication to alleviate symptoms and slow progression of the disease. Treatment can prove financially burdensome, with medication alone costing upwards of $30,000 per year and RA-associated treatment costs in the United States totaling $19 billion annually (2–4). A 2015 retrospective study found that people with RA miss an average of 4 more days of work per year than those without the disease, resulting in an estimated $252 million in lost revenue every year due to RA-related work absences (5). Collectively, the steadily increasing occurrence, high treatment costs, and the significant economic burden associated with RA highlight the importance of developing better prevention, detection, and treatment methods. This introduction is going to outline the disease pathogenesis, genetic risk factors, and conclude with environmental risk factors including the potential role of microbial involvement and disease progression, which is the focus of my research.

Disease Pathogenesis

Rheumatoid arthritis results when an individual’s immune system produces autoreactive T cells and autoantibodies that attack synovial joint tissues, tendons, and bursae. This generates a potent inflammatory response that results in joint swelling, tenderness, stiffness, and pain. Left untreated, this chronic inflammation can lead to irreversible damage to cartilage and bones of the afflicted joint and an eventual loss of
mobility, functionality, and quality of life (6). Permanent joint damage, including erosion of bones, thinning of cartilage, loss of structural integrity, and loss of bone density are observed in the majority of RA patients within two years of the onset of symptoms (7, 8). Thus, early diagnosis and intervention is essential to prevent permanent damage, with the optimal treatment window being within 3-6 months following the onset of symptoms (9).

In the preclinical stages of RA, epigenetic and environmental factors disrupt cytokine homeostasis. Increased levels of circulating proinflammatory cytokines, including TNF, IL-6, IL-1, granulocyte-macrophage-colony stimulating factor (GM-CSF), as well as increases in IL-5, IL-12, IL-17, and eotaxin (a chemokine specific to eosinophils) have been observed. These cytokines arise from a number of different sources, including the affected joint itself or the periphery (10). One particularly important cytokine is TNF, which has been implicated in the transition from preclinical stages to chronic synovitis through promoting osteoclast, chondrocyte, vascular endothelium, and fibroblast activation. Additionally, TNF promotes increased production of localized proinflammatory cytokines, including IL-1, IL-6, and GM-CSF by monocytes and macrophages (11, 12).

Increased expression of proinflammatory cytokines in the synovium in turn facilitates invasion of fibroblast-like synoviocytes and neutrophils that release chemokines that attract Th17 lymphocytes (12). Th17 lymphocytes then produce proinflammatory cytokines IL-17 and IL-22, which trigger additional inflammatory responses and recruitment of immune cells (13). Steady accumulation of immune cells results in swelling of the affected joints and promotes angiogenesis of synovial vasculature. Angiogenesis helps facilitate cellular invasion deeper into joint tissues, as
the newly formed vasculature lacks structural integrity and leaks leukocytes into the surrounding tissues. Repeated angiogenesis and accumulation of tissue infiltrates can result in the formation of an abnormal invasive tissue known as pannus within the synovium. As pannus thickens, it can extend into synovial tissues, bones, and cartilage and cause irreversible bone and muscle damage (12, 14).

**Citrullination and Rheumatoid Arthritis**

Anti-citrullinated peptide antibodies (ACPAs) are autoantibodies specific to self-proteins that have been post-translationally modified through citrullination. During the process of citrullination, arginine is deiminated and converted into citrulline, an amino acid that is not part of protein synthesis when mRNA is first translated, as shown in figure 1 (15, 16).

![Citrullination Reaction](https://www.cytoskeleton.com/blog/citrullination-news-detailed)

This reaction is driven by the activation of intracellular catalytic enzymes known as peptidylarginine deiminases (PADs), which are calcium-dependent enzymes found in epidermal tissues, skeletal muscles, neutrophils, eosinophils, fibroblast-like synoviocytes, monocytes, natural killer cells, and macrophages (17–20). Conversion of arginine to
citrulline results in a loss of charge, as arginine is positively charged and citrulline has no net charge. Loss of charge and resultant changes in hydrophobicity have the potential to induce conformational changes in citrullinated peptides that ultimately impact the structure and function of the protein. Such changes also have the potential to increase the immunogenicity of peptides by revealing novel binding epitopes (15, 16). PAD enzymes are not found in the thymus so citrullination does not occur there thus, T-lymphocytes with an affinity for citrullinated peptides are not deleted during negative selection. Such lymphocytes can become autoreactive following citrullination of self-peptides, subsequently inducing the production of ACPAs by B cells (18, 21, 22).

Citrullination is observed at higher frequencies during infectious and inflammatory processes of various tissues, including the lungs, central nervous system, epidermis, and skeletal muscles (23). In RA patients, inflammatory infiltrates and fibroblast-like synoviocytes express increased levels of PAD-2 and PAD-4, suggesting a predisposition to citrullination of synovial peptides (18). This is supported by previous studies, which have demonstrated elevated levels of citrullinated vimentin, fibrin, fibrinogen, enolase, and histone H2B within the synovium of RA patients (16, 24–26). Citrullinated synovial peptides can then become the target of ACPAs produced in the periphery in response to inflammatory events triggered by environmental factors, like dental infections and cigarette smoke (27).

During the preclinical phase of RA, increased levels of circulating TNF and IL-6 correlate with diversification of specificity, enhanced binding avidity, and class switching of ACPA IgM to IgA and IgG isotypes, all of which can increase the pathogenicity of ACPAs and their reactivity with citrullinated peptides. (18, 28). This is supported by
previous studies demonstrating that sera from RA patients, but not healthy patients, react with citrullinated epitopes of cytoskeletal proteins such as vimentin, fibrin, fibrinogen, collagen, and histone H2B (29). Upon binding to citrullinated synovial proteins, ACPAs can initiate an inflammatory cascade, inducing synovitis and the formation abnormal synovial tissue known as pannus (18).

An estimated 67% of RA patients show serologic evidence of ACPA production, which is associated with severe symptomology (30). As these antibodies have been detected in some patients up to 10 years before the onset of symptomology, the presence of ACPAs in patient serum is considered one of the best biomarkers for early detection of RA (7). During the early stages of RA, ACPAs have been detected in synovial joints and epithelial sites of RA patients (22).

Genetic Risk Factors

Many different genetic risk factors render individuals susceptible to the production of autoreactive antibodies, although it is important to note that the risk factors differ between ACPA seropositive and seronegative adults (30). Genetic risk factors are polygenic, with over 100 different loci implicated to date (31, 32). In general, through studying incidence of RA in identical twins and genome-wide association studies, it is estimated that RA is 65% heritable, independent of age, age at onset, and morbidity (33).

HLA Genes.

Allelic variation of genes associated with the major histocompatibility complex (MHC) region have been implicated in the pathogenesis of over 100 autoimmune diseases, including ACPA seropositive RA. Nearly 60% of RA heritability is linked to MHC allelic variations (31, 32). This locus is responsible for production of human
leukocyte antigens (HLAs). Class II MHC molecules present processed which present processed antigen peptides to T-helper cells to initiate an adaptive immune response (34).

Of all the genes involved in MHC formation, the most commonly recognized gene associated with the pathogenesis of RA is the \textit{HLA-DRB1} gene, which encodes the \( \beta \)-chain of DR MHC II molecules. The \( \beta \)-chain complexes with the \( \alpha \)-chain (encoded by \textit{HLA-DRA}), to form a functional protein responsible for presentation of peptides to T-helper cells (31, 34). Hundreds of different allelic variants of \textit{HLA-DRB1} exist. Alleles containing shared epitopes (SE) QKRAA, QQRAA and KKRAA at amino acids 70 to 74 in the third hypervariable region of \textit{HLA-DRB}, which are located in the peptide-binding groove, are strongly associated with the production of ACPAs (29). Patients with allelic variants containing risk-associated SEs have been shown to produce elevated amounts of ACPAs specific to synovial proteins such as vimentin, fibrinogen, enolase, and myelin basic protein. In contrast, alleles containing the amino acid binding epitope DERAA at positions 70-74 affords some protection against ACPA+ RA (31, 32, 35, 36). The “shared-epitope hypothesis” proposes that certain allelic variants of HLA more readily present citrullinated peptides, rendering individuals with such allelic variants genetically predisposed to the production of ACPAs following inflammatory events that induce citrullination of self-tissues (37).

Several HLA-associated single nucleotide polymorphisms (SNPs) that confer increased susceptibility to seropositive RA have been identified using genome-wide analysis studies. The presence of valine at amino acid position 11 and histidine at amino position 13 of HLA-DRB1 and SNPs at amino acid position 9, 71, and 74 of HLA-DRB1, and amino acid position 9 of HLA-B of MHC I molecules all have strong associations
with the ACPA-seropositive RA. The presence of serine at amino acid position 11 of HLA-DRB1 has been shown to afford protection against development of ACPA seropositive RA but has a strong association with the development of seronegative RA. It is hypothesized that conformational changes induced by SNPs impact the peptide-binding affinity and can enhance the recognition of citrullinated autoantigens and subsequent formation of ACPAs (31, 32) A summary of HLA-DRB1 allelic variants and their association with RA can be found in Table 1.

Table 1. Allelic variants of HLA-DRB1 and their association with seropositive RA (38)
Non-HLA Genes.

A number of non-HLA genes have also been identified as potential risk factors for RA, with the majority of the loci being implicated in other autoimmune diseases. Nearly 66% of RA cases are observed in women, suggesting involvement of X-linked genetic factors in the pathogenesis of RA (36, 39). Most other variants linked to RA involve SNPs within immunoregulatory genes, such as the promoter region of genes responsible for cytokine production (35). Polymorphisms in the promoter region for the genes encoding the pro-inflammatory cytokine IL-1β and the immunoregulatory and anti-inflammatory cytokine IL-10 have been linked to the onset and progression of RA (36). Such mutations have the potential to induce upregulation of these cytokines, resulting in loss of regulatory control of inflammatory processes and subsequently contribute to the onset and pathology of RA (31). Mutations in PADI4, which encodes PAD-4, have also been associated with RA, as they can render an individual more susceptible to citrullination (35).

Although a number of polymorphisms are associated with both seropositive and seronegative individuals, certain polymorphisms are only associated with one serostatus (32, 35). Examples include missense mutations in PTPN22, which encodes for a protein involved in the development and activation of lymphocytes, tolerance, and regulation of immune modulation (40). Such mutations are observed at an elevated frequency in seropositive RA patients, but not seronegative patients. SNPs in ANKRD55, which encodes a protein of unknown function, are also the only genome-wide markers associated with RA susceptibility in seronegative individuals (32, 41). This gene has also
been implicated in a number of other autoimmune diseases, including multiple sclerosis, Crohn’s disease, celiac disease, and type 1 diabetes (41).

Certain SNP’s near the ANKRD55 locus have also been shown to indirectly impact the glycosylation patterns of the Fc region of human IgG. As the glycosylation patterns of this region are ultimately responsible for antibody effector function, it is possible that these SNP’s alter IgG effector function enough to promote the formation of autoantibodies and subsequent onset of autoimmune disease. In RA patients, the absence of galactose in the Fc region of human IgG has a strong association with pro-inflammatory IgG phenotypes observed during RA-associated inflammation, whereas the presence of sialic acid in this region is associated with an anti-inflammatory IgG phenotype observed during remission (42).

**Environmental Risk Factors**

Despite the fact that many allelic variants have been linked to RA, the presence of allelic risk factors alone is not enough to trigger the expansion of autoreactive immune cell (36). The pathogenesis of RA is complex and has been described as a “Bermuda triangle” because the disease involves a complex interplay between autoreactive immune cells, genetic predisposition, and environmental/lifestyle-related events that trigger an autoimmune reaction (35).

**Cigarette Smoke.**

Smoking is considered one of the strongest environmental risk factors associated with the production of ACPA and the eventual development of RA, although the mechanisms through which smoking might lead to the onset of RA are not well understood (35, 36). Chronic exposure to cigarette smoke has been shown to increase
expression of PAD enzymes by epithelial and phagocytic cells of the lungs, promoting
citrullination of lung tissues. In those with allelic variants of HLA-DRB1 that contain the
SE, increased citrullination can result in the production of ACPAs specific to citrullinated
lung tissues (43, 44).

Rat models have demonstrated that following recognition of citrullinated lung
peptides, activated T cells travel to bronchus-associated lymphoid tissue through draining
lymph nodes and reenter blood circulation. During this migration, T cells undergo
epigenetic modifications that upregulate expression of chemokine receptors and cellular
adhesion molecules, promoting migration toward inflamed tissues and affording the
capacity to breach epithelial barriers. Thus, it is possible that autoreactive T cells
produced in response to citrullination of lung peptides could migrate to the synovium
(45).

Studies have shown that smokers carrying allelic variants of HLA-DRB1 that
contain the SE have a 21-fold higher risk of developing ACPA seropositive RA than non-
smokers whose MHC alleles do not contain the SE. This demonstrates the interplay
between genetic predisposition and environmental risk factors involved in RA (46).

**Microbes as Risk Factors**

Microbes have the potential to trigger the onset and facilitate the progression of
RA through a number of different mechanisms, the first of which is known as “molecular
mimicry” (36). This concept hypothesizes that a cellular immune response mounted
toward a microbial peptide that closely resembles a self-peptide has the potential to form
cross-reactive B and T lymphocytes, effectively promoting the onset of RA through loss
of immunologic tolerance. Bacterial, viral, and fungal infections have the potential to
trigger numerous different T-cell specific autoimmune diseases through molecular mimicry (47). Molecular mimicry is exemplified by bacterial heat shock protein (Hsp40). Previous studies have illustrated that anti-bacterial Hsp40 antibodies are cross-reactive with human Hsp40. Studies have noted that RA patients have elevated titers of anti-bacterial Hsp40, as well as anti-human Hsp40 antibodies. Collectively, this suggests the possibility that cross-reactivity between bacterial and human anti-Hsp40 antibodies can act as a triggering event the production of autoreactive immune cells and development of autoimmune disease (47, 48).

In addition to molecular mimicry, microbial peptides can drive autoimmunity via activation of pattern recognition receptors that drive the production of RA-associated cytokines, such as IL-1, IL-6 and IL-33 (49). Interleukin-1 is involved in the pathogenesis of RA, as it triggers fibroblast proliferation, which drives the formation of pannus in the synovium. Additionally, IL-1 activates chondrocytes and osteoclasts, which are involved in cartilage degradation and bone resorption respectively (50). Microbial infections also have the potential to activate monocytes and neutrophils expressing high levels of PAD enzymes, which can drive the citrullination of self-peptides and potentially result in ACPA production in genetically predisposed individuals. A number of different dental, respiratory, and urinary-tract pathogens have been associated with the pathogenesis of RA (49).

Dental Infections.

Periodontitis (PD), a common comorbidity associated with RA, is observed in nearly 54% of patients with RA compared to 8% of those without (51). Periodontitis is an inflammatory disease of the tissues, bones, and ligaments encasing teeth that results from
dysbiosis of oral microbiota. Smoking is considered one of the biggest risk factors associated with developing PD (52). The pathogenesis of PD is similar to that of RA, and results in chronic inflammation and degradation of the bones and tissues of the periodontium. High levels of ACPAs have been observed in non-RA patients with PD, suggesting that PD has the potential to trigger a loss of tolerance and subsequent production of autoreactive ACPAs (53).

Rheumatoid arthritis patients have significantly higher serum and synovial fluid IgG antibody titers specific to Gram-negative bacteria compared to healthy controls, providing evidence to support the link between dental infections and RA (52).

*Porphyromonas gingivalis*, an anaerobic Gram-negative coccobacillus, is one of most common causes of PD (54). *P. gingivalis* has been directly implicated in the breakdown of immunologic tolerance and subsequent production of ACPAs through a variety of different mechanisms, suggesting that it could also drive the onset of RA. *P. gingivalis* encodes its own PAD enzyme, which can promote citrullination of self-peptides of the periodontium. Subsequently, this bacterium may promote the production of ACPAs, which can result in a breakdown in tolerance in those carrying allelic variants of *HLA-DRB1* that contain the SE. Neutrophils recruited to the site of *P. gingivalis* infection release additional PADs and damage-associated molecular patterns, which drive both localized and systemic inflammatory responses, as well as the production of ACPAs. Additionally, *P. gingivalis* contains pathogen-associated molecular patterns, such as lipopolysaccharide (LPS), which are capable of triggering signaling and inflammatory-cytokine cascades via pattern-recognition receptors (53). Collectively, the mechanisms by which *P. gingivalis* can promote loss of tolerance highlights the extent to which microbial
infections at an epithelial site can drive the production of autoreactive antibodies involved in the pathogenesis of RA (53).

**Dysbiosis of Gut Microbiota.**

Gut dysbiosis has also been implicated in loss of tolerance in individuals genetically predisposed to RA (55). The human gut microbiota is composed of a diverse group of microbes including viruses, fungi, protozoa, and between 500-1,000 different species of bacteria (56–58). Collectively, this community is involved in many different metabolic processes, such as the production of enzymes that increase the rate of polysaccharide metabolism. Additionally, gut microbiota are also involved in many physiological processes, like gut epithelial development, lymphocyte production, and angiogenesis (59). In addition to metabolic and physiologic involvement, the gut microbiota is considered a first-line of defense in innate immunity and provides immunologic protection from foreign microbes in several different ways (57). For example, the large abundance of commensal bacteria of the gut reduce attachment sites available and rapidly depletes any energy sources available for pathogenic organisms. The gut microbiota has also been shown to produce antimicrobial peptides in addition to inducing a host antimicrobial peptide response, another important way that the gut microbiota is involved in immune function (60).

For proper physiological and metabolic function, the intestinal immune system must maintain a symbiotic relationship with the gut microbiota. For this relationship to remain beneficial, the intestinal immune system must protect the host from frequent exposure to pathogenic organisms found in food and water while avoiding excessive responses toward many different food proteins (59). Under certain environmental
conditions, such as dietary changes, smoking, alcohol consumption, or antibiotic usage, some bacterial species of gut microbiota either increase or decrease in quantity (61, 62). Fluctuations in the composition of the gastrointestinal microbiota has been associated with RA (63).

Murine models have shown that certain gut pathobionts, like Enterococcus gallinarum, have the potential to translocate from the gut epithelia to other systemic organs following disruptions in the gut microbiota. Colonization of germ-free mice with E. gallinarum resulted in downregulation of proteins involved in maintenance of a healthy gut barrier in addition to upregulated expression of proteins related to inflammatory processes, including AhR, which is a known inducer of proinflammatory Th17 cellular responses. Genome-wide association studies have revealed that E. gallinarum encodes several natural AhR ligands, suggesting that translocation has the potential to drive autoimmune response in genetically predisposed individuals through directing T cell differentiation toward a proinflammatory Th17 response (64).

**Synovial Pathobionts and RA.**

Traditionally, the synovial space in joints has been considered sterile, except in the case of septic arthritis (63). However, the inflamed environment of the synovium has been shown to trap components of pathobionts that have translocated to the synovium following dysbiosis of epithelial normal flora, effectively creating a pocket of concentrated immunogenic material that can maintain an active antibody response in synovial tissues (65, 66). Trapped bacterial components also have the potential to induce inflammatory cascades through shedding of immunogenic substances that activate toll-
like receptors (TLRs), such as TLR3 or TLR9 via recognition of bacterial RNA or DNA, respectively (63).

For example, a recent study published in PLOS reported the presence of bacterial DNA in the synovial fluids of both RA patients and healthy controls, suggested the possibility of a previously uncultured synovial microbiome or translocation of microbial DNA. Comparison of 16 RA patients and 10 normal healthy donors (NHD) using 16S rRNA gene sequencing revealed the presence of several bacteria that are differentially present between RA patients and healthy controls. Despite no previous association with RA, Gram-negative bacteria from the genus *Raoultella* were found almost exclusively in the synovial fluid and tissues of RA patients and accounted for a 6.5% of the bacterial community, suggesting possible involvement in RA (63). Two members of this genus, *R. planticola* and *R. ornitholytica* are pathogenic to humans (67).

*Raoultella* species were previously classified under the genus *Klebsiella*. *Raoultella* is most commonly isolated from environmental sources such as plants, water, and soil. There are four species in the genus—*R. planticola*, *R. ornitholytica*, *R. terrigena*, and *R. electrica*. All are aerobic non-motile rods that are catalase positive, ferment lactose and glucose, and can reduce nitrate to nitrite. In humans, *Raoultella* colonizes the respiratory and gastrointestinal tract of an estimated 9% of newborns (67). Both *R. planticola* and *R. ornitholytica* are opportunistic pathogens that have been documented in a small number of cases of bacteremia, pneumonia, and urinary, gastrointestinal, surgical wound, and dental infections (68, 69). Virulence factors include the production of a polysaccharide capsule, fimbriae, lipopolysaccharides, siderophores, bacteriocins, and the ability to form biofilms (67).
OBJECTIVES

Detection of Human Serum Antibodies Specific-to Raoultella

The report described above, that correlated the presence of Raoultella with RA only included 16 RA patients and 10 NHD (63). Therefore, it is important to expand the number of patients tested to better support a possible association between Raoultella and RA. Collection of synovial fluid is invasive and not routinely performed on RA patients or NHD. Therefore, a minimally invasive laboratory test capable of detecting Raoultella-specific serum antibodies to investigate the association of Raoultella and RA would be advantageous. Additionally, a serum antibody test can provide evidence of past infections with microbes that may have played a role in disease onset, but are no longer present. Finally, such antibodies may play a direct role in, or serve as an indicator of pathogenic B and T cell responses associated with the immune pathology of RA.

Several factors complicate the development of an accurate and reliable genus-specific serologic antibody test. The first of these is that bacteria induce a polyclonal B cell response, effectively resulting in the production of antibodies capable of recognizing antigens shared among related species (70). Therefore, attention must be paid to identifying genus-specific antibody responses. Additionally, serum immunoglobulins can interact non-specifically with the target antigen or blocking buffer components via non-covalent protein-protein interactions. This propensity varies from patient-to-patient and is increased in RA patients compared to NHD, which can lead to false positive serum antibody test results. Therefore, it is important to use a pre-absorptive blocking agent that effectively eliminates non-specific antibody interactions (71).
If Raoultella-specific antibody differences are observed between RA patients and NHD, this test has the potential to improve our understanding of RA pathology and current diagnostic capabilities. The overall goal of this project was to develop an enzyme-linked immunosorbent assay (ELISA) capable of detecting whether there is a significant difference in Raoultella-specific serum antibody titers between RA patients and NHD with a high degree of sensitivity.

Aim 1: Establish pure cultures of R. ornitholytica, R. planticola, and S. pneumoniae (human serum antibody control)

Aim 2: Generate bacterial whole-cell lysate for each cultured organism

Aim 3: Develop an ELISA to screen human serum for antibodies reactive with Raoultella antigens in bacterial whole-cell lysates
MATERIALS AND METHODS

Aim 1: Establish Pure Cultures of *R. ornitholytica*, *R. planticola*, and *S. pneumoniae*

Media Preparation.

Bacterial culture media was prepared using the recipes found in Table 2.

<table>
<thead>
<tr>
<th>Culture Media</th>
<th>Composition/L water*</th>
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<tr>
<td>Nutrient broth</td>
<td>5 g gelatin peptone, 3 g beef extract</td>
</tr>
<tr>
<td>Nutrient agar</td>
<td>5 g gelatin peptone, 3 g beef extract, 120 g gelatin</td>
</tr>
<tr>
<td>Tryptic soy broth (TSB)</td>
<td>20 g non-animal peptone, 2.5 g D(+) glucose, 5 g sodium chloride, 5 g dipotassium hydrogen phosphate</td>
</tr>
<tr>
<td>Tryptic soy agar (TSA)</td>
<td>15 g pancreatic digest of casein, 5 g papainic digest of soybean, 5 g sodium chloride, 15 g agar</td>
</tr>
<tr>
<td>5% sheep blood agar (SBA)</td>
<td>Purchased from Becton Dickinson BBL™</td>
</tr>
</tbody>
</table>

*Heated to dissolve and autoclaved for 15 minutes at 121° C.

Cultivation of Bacteria.

Freeze-dried cultures of *R. ornitholytica* and *R. planticola* were purchased from American Type Culture Collection (ATCC). A fresh culture of *Streptococcus pneumoniae* was obtained from the Microbiology prep room at UW-La Crosse.

*R. ornitholytica* (ATCC 31898) was cultured using ATCC recommendations.

Freeze-dried culture was reconstituted in nutrient broth, streaked onto a nutrient agar plate, and incubated aerobically at 37°C for 24-48 hours.
Isolated colonies were picked and used to inoculate nutrient broth.

*R. planticola* (ATCC 21534) was cultured using ATCC recommendations. Freezedried culture was reconstituted in TSB, streaked onto TSA, and incubated aerobically at 26°C for 24-48 hours. Isolated colonies were picked and used to inoculate TSB.

To culture *S. pneumoniae*, stock culture was streaked onto 5% SBA and incubated in a candle jar at 37°C for 24-48 hours until growth of the bacterium was observed. After growth was observed on SBA, isolated colonies were picked and used to inoculate TSB.

**Aim 2: Generating Whole Cell Lysates**

Whole cell lysates were generated for each bacterial species. For each organism, turbid broth cultures (incubated 24-48 hours), were pooled and centrifuged at 4000 x g for 5 min. After discarding the supernatant, the pellet was resuspended in phosphate-buffered saline (PBS) and centrifuged at 14,000 x g for 5 min.

Each pellet was washed twice more, resuspended in PBS, and transferred to lysing matrix E bead beating tubes purchased from MP Biomedicals. Bacteria were subjected to 6 min of bead beating, while being placed on ice every 2 minutes to avoid denaturation of macromolecules caused by heat produced during bead beating (72). After bead beating, tube contents were centrifuged at 14,000 x g for 5 minutes to separate supernatant from the lysing beads. Supernatant containing whole cell lysate was harvested, pooled, and stored at 4°C.
**Lysate Protein Concentration Quantification.**

A Pierce bicinchoninic acid (BCA) protein assay kit was used to quantify the concentration of protein in each bacterial lysate ([https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011430_Pierce_BCA_Protein_Asy_UG.pdf](https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0011430_Pierce_BCA_Protein_Asy_UG.pdf)).

**Aim 3: Develop an ELISA to Screen Human Serum for Antibodies Reactive with *Raoultella* Antigens in Whole Cell Lysates**

**Human Serum Samples.**

Nine serum samples collected from patients with clinically diagnosed RA were purchased from ProMedDx biospecimen services. Serum previously collected from a UWL faculty member, who has clinically diagnosed RA, served as a tenth RA sample. Five serum samples collected from current and past laboratory members free of any clinically diagnosed autoimmune diseases served as NHDs.
Table 3. Case report information on RA serum samples

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Clinical Diagnosis (Autoimmune)</th>
<th>Concurrent Medical Conditions / Clinical Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>52</td>
<td>F</td>
<td>RA</td>
<td>Hypertension, Irritable bowel syndrome, Depression, PAI-1 (high clot risk), Hyperhomocysteinemia</td>
</tr>
<tr>
<td>65</td>
<td>F</td>
<td>RA</td>
<td>Osteoporosis, Rheumatoid Arthritis, Vaginal dryness</td>
</tr>
<tr>
<td>65</td>
<td>F</td>
<td>RA</td>
<td>Fatigue, Obesity, Arthritis, Rheumatoid arthritis, Type II Diabetes, Sleep apnea, Pain all over, Allergies, Myalgia</td>
</tr>
<tr>
<td>53</td>
<td>F</td>
<td>RA</td>
<td>Hyperlipidemia/Dyslipidemia, Hypothyroidism, Headaches, Joint pain, Restless leg syndrome, Vitamin D deficiency</td>
</tr>
<tr>
<td>36</td>
<td>M</td>
<td>RA</td>
<td>None</td>
</tr>
<tr>
<td>61</td>
<td>F</td>
<td>RA</td>
<td>Anxiety Disorder, Asthma, Gastroesophageal Reflux Disease (GERD), Hypothyroidism, Rheumatoid Arthritis</td>
</tr>
<tr>
<td>85</td>
<td>F</td>
<td>RA</td>
<td>Arthritis, Hypertension, Hypothyroidism, Osteoporosis, Osteoarthritis, Gallbladder disease, History of blood clots, Rheumatoid Arthritis</td>
</tr>
<tr>
<td>80</td>
<td>F</td>
<td>RA</td>
<td>Arthritis, Edema, Gastroesophageal Reflux Disease (GERD), Hypothyroidism, Joint pain, Rheumatoid Arthritis, Vertigo</td>
</tr>
<tr>
<td>67</td>
<td>F</td>
<td>RA</td>
<td>Anemia, Arthritis, Dry Eyes, Gastroesophageal Reflux Disease (GERD), Headaches, Hypertension, Osteopenia, Shoulder pain, Joint pain, Low back pain, Restless leg syndrome, Rheumatoid Arthritis, Vitamin D deficiency</td>
</tr>
<tr>
<td>57*</td>
<td>F</td>
<td>RA</td>
<td>Rheumatoid Arthritis, Hypothyroidism, Vitamin D deficiency, Allergies, Hyperlipidemia</td>
</tr>
</tbody>
</table>

*Collected from UW-La Crosse faculty member. All other samples purchased from ProMedDx
**ELISA Protocol.**

Off-target binding of serum immunoglobulins to shared epitopes of bacterial or blocking buffer proteins and a variety of other non-specific reactions, such as non-antigen-binding protein-protein interactions, have the potential to cause false positives during an indirect ELISA (73). Consequently, a blocking system that effectively minimizes these non-specific reactions is essential to obtain accurate and interpretable serologic data via indirect ELISA. ChonBlock™, a mammalian protein-based ELISA blocking buffer manufactured by Chondrex, Inc (a company specializing in RA research products), was utilized, as previous studies show that it effectively eliminates background noise and off-target immunoglobulin reactions, leading to collection of data that is more accurate, reliable, and easily interpreted (71).

The protocol provided with the ChonBlock™ ELISA buffer system was used for all ELISAs (https://www.chondrex.com/documents/ChonBlock-Protocol.pdf). A 96-well polystyrene plate was coated with bacterial whole-cell lysate. After incubating at 4°C for 24 hours and washing three times with distilled water, ChonBlock™ blocking/sample dilution buffer was added to each well. All subsequent incubations were performed at room temp and all washes were performed three times using PBS containing 0.05% Tween 20. After incubating for one hour, the plate was washed. In duplicate, two-fold dilutions of serum samples diluted in blocking/sample buffer, from a dilution of 1:100 to 1:12,800 was added. After two hours of incubation the plate was washed. Antigen-specific antibodies were detected by adding goat anti-human IgG (H+L) conjugated to horseradish peroxidase (HRP), diluted in ChonBlock™ secondary antibody dilution
buffer. Following an hour-long incubation and washing, chromogenic substrate tetramethylbenzidine (TMB) was added to each well, in order to detect the binding of detection antibody via a colorimetric reaction. After 15 minutes of incubation, stop solution (2N sulfuric acid) was added to each well to quench the colorimetric reaction and optical density (OD) was measured at 450 nm.

All ELISAs included the controls shown in Table 4. *Streptococcus pneumoniae* was chosen as a positive control because no true positive antibody control serum, i.e. human serum containing *Raoultella*-specific antibodies, is available for either *Raoultella* species. This species allowed for verification of the indirect ELISA’s ability to detect human serum antibodies because with few exceptions, all humans have naturally acquired adaptive immunity to *S. pneumoniae* (74).

<table>
<thead>
<tr>
<th>Purpose of Control</th>
<th>Coat</th>
<th>Block</th>
<th>Serum</th>
<th>Secondary Antibody</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control:</td>
<td><em>S. pneumoniae</em></td>
<td>yes</td>
<td>Yes (1:100 dilution)</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Verify ELISA detects species-specific antibodies in human serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative control:</td>
<td>None</td>
<td>yes</td>
<td>Yes (1:100 dilution)</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Determine OD of human serum antibodies binding to the block</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>None</td>
<td>Yes</td>
<td>No</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Determine OD of block in absence of antibodies</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Controls included in each ELISA
Optimization of ELISA by Checkboard Titrations.

To optimize the concentration of bacterial whole-cell lysate and secondary antibody dilution, a checkerboard ELISA was performed. Two-fold dilutions of *S. pneumoniae* whole cell lysate were performed vertically down the plate, from a concentration of 20 μg/ml to a concentration of 1.25 μg/ml. An RA patient serum at a 1:500 dilution was used. In duplicate, two-fold dilutions of secondary antibody (goat anti-human IgG (H+L) conjugated to HRP) were plated horizontally across the plate from a dilution of 1:2,500 to 1:20,000.

To verify that the optimization of whole cell lysate and secondary antibody extends to *Raoultella* species, a checkerboard titration was performed for *R. planticola* and *R. ornitholytica*. The plate was coated with a two-fold dilution series of whole cell lysate, from a concentration of 20 μg/ml to a concentration of 1.25 μg/ml. RA patient serum at a concentration of 1:100 was used. Secondary antibody was used at the optimal dilution, determined previously by the *S. pneumoniae* checkerboard titration.

Screening RA and NHD Serum.

After optimization of whole cell lysate concentration and secondary antibody dilution, ELISAs were performed using two-fold dilutions from 1:100 to 1:12,800 of the human serum samples (in duplicate), screening against plates coated with 1 μg/ml of *R. planticola, R. ornitholytica, or S. pneumoniae* whole-cell lysates.

Statistical Analysis.

To determine statistical significance, a Welch’s t-test was used (75). The blank-subtracted average OD of the optimal serum dilution for RA patients (X1) and NHD patients (X2) was calculated. Next, standard deviation for RA patients (SD1) and NHD
(SD2) was calculated. Taking into account the number of RA patients (n1) and NHD (n2) included, the following equation was used to calculate t-value:

$$ t = \frac{X_1 - X_2}{\sqrt{\frac{SD_1^2}{n_1} + \frac{SD_2^2}{n_2}}} $$

After calculating t-value, degrees of freedom (ν) were calculated using the following formula:

$$ \nu = \frac{(SD_1 + SD_2)^2}{\left(\frac{(SD_1)^2}{n_1 - 1} + \frac{(SD_2)^2}{n_2 - 1}\right)} $$

After calculating degrees of freedom and rounding down to the next integer, the critical t-value was obtained from a t-distribution table, with a confidence level of 95%. If the calculated t-value was less or equal to the critical t-value, no significant difference existed between the OD of RA patients and NHD.

**RESULTS**

**Establishment of Pure Bacterial Cultures**

Colony morphology and Gram staining of each culture matched established characteristics for each organism and no contamination was detected (Table 5). Species identity for each culture was confirmed using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) at Gundersen Health System. Turbid broth cultures were grown successfully from isolated colonies of each organism, providing bacterial cells for generating bacterial whole-cell lysates.
Table 5. Colony morphology and Gram stain of each cultivated species used to verify pure culture.

<table>
<thead>
<tr>
<th>Species</th>
<th>Plate Media</th>
<th>Colony Morphology</th>
<th>Gram Stain</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. pneumoniae</em></td>
<td>5% SBA</td>
<td>small white mucoid colonies, α-hemolysis</td>
<td>Gram positive lancet-shaped diplococci</td>
</tr>
<tr>
<td><em>R. ornitholytica</em></td>
<td>Nutrient agar</td>
<td>Small off-white opaque colonies</td>
<td>Gram negative rods</td>
</tr>
<tr>
<td><em>R. planticola</em></td>
<td>TSA</td>
<td>Small off-white opaque colonies</td>
<td>Gram negative rods</td>
</tr>
</tbody>
</table>

**Generation of Bacterial Whole-Cell Lysate**

Whole cell lysates were generated for each organism using pure broth cultures of each organism. The protein concentration of pooled lysate, for each organism, determined using a BCA assay, is shown in Table 6.

Table 6. Whole cell lysate protein concentrations obtained through bead beating, measured using the BCA protein assay. Lysates were prepared in excess to ensure the same lysate sample was used for every ELISA.

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. pneumoniae</em></td>
<td>207</td>
</tr>
<tr>
<td><em>R. ornitholytica</em></td>
<td>91</td>
</tr>
<tr>
<td><em>R. planticola</em></td>
<td>214</td>
</tr>
</tbody>
</table>

**ELISA Optimization**

Optimization of Whole Cell Lysate and Secondary Antibody.
OD values relative to *S. pneumoniae* lysate concentrations were more or less plateaued across all lysate concentrations from 1.25 µg/ml to 20 µg/ml (Figure 3). OD values relative to secondary antibody dilution were highest at 1:2,500, followed by 1:5,000, with the OD value at 1:10,000 and 1:20,000 being substantially lower (>1.0). Due to a need to conserve reagents, while ensuring that any differences in OD values remain detectable between RA and NHD serum, a 1 µg/ml bacterial whole cell lysate coating concentration was chosen to coat each well and a 1:5,000 dilution of secondary antibody was chosen as the secondary antibody dilution.

Figure 2. Two-fold dilutions of *S. pneumoniae* whole cell lysate at a concentration of 20 µg/ml to a concentration of 1.25 µg/ml were used to coat wells. A randomly selected RA serum sample was diluted 1:500. Two-fold dilutions of secondary antibody from a dilution of 1:2,500 to 1:20,000 were tested at each lysate concentration.

The checkerboard ELISA performed on each *Raoultella* species verified that whole cell lysate at a concentration of 1.0 µg/ml and secondary antibody diluted 1:5,000
(as determined in Figure 2) provided a strong measurable OD value for each Raoultella species (Figure 3).

Figure 3. Two-fold dilutions of Raoultella whole cell lysate in PBS were performed from a concentration of 5.0 µg/ml to 0.312 µg/ml. A randomly selected RA serum sample was diluted 1:100 and secondary antibody was diluted to 1:5,000. This assay verified that optimal concentrations determined via S. pneumoniae checkerboard titration extended to Raoultella species.

**Optimization of Human Serum Dilutions.**

Screening human serum at various dilutions against a 1.0 µg/ml, with secondary antibody diluted 1:5,000, revealed that a 1:400 serum dilution provided a measurable difference between RA and NHD human serum samples (Figure 4).
Figure 4: Wells were coated with *R. ornitholytica* or *R. planticola* whole cell lysate at a concentration of 1.0 µg/ml. Two-fold dilutions of a randomly selected RA and NHD serum sample were performed from 1:100 to 1:12,800. Secondary antibody was diluted 1:5,000.

OD values of each control from a representative plate are shown in Table 7.

Table 7: OD values of each control from a representative plate are shown. OD was measured at 450 nm. These controls were run on each ELISA

<table>
<thead>
<tr>
<th>Coat</th>
<th>Block</th>
<th>Serum</th>
<th>Secondary Antibody</th>
<th>Substrate</th>
<th>Mean RA OD (450 nm)</th>
<th>Mean NHD OD (450 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. pneumoniae</em></td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>3.768</td>
<td>3.876</td>
</tr>
<tr>
<td>None</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>0.029</td>
<td>0.008</td>
</tr>
<tr>
<td>None</td>
<td>Yes</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Screening RA and NHD Serum.

All serum samples were screened against *Raoulittella* species at a 1:400. *S. pneumoniae* was screened against human serum diluted 1:100, for ease of dilution and to ensure that blocking and buffering components were not saturated, which could lead to
false positive results. Using the optimized ELISA, all human serum samples were screened against whole cell lysate from each bacterial species (Figures 5).

Figure 5: mean OD values at 450 nm from indirect ELISA screening of RA and NHD serum. Wells were coated with *R. ornitholytica* or *R. planticola* whole cell lysate at a concentration of 1.0 µg/ml and secondary antibody was diluted 1:5,000. Results from human serum diluted 1:400 are plotted, as results in figure 5 indicated that a 1:400 dilution provided a measurable difference between RA and NHD human serum samples. The whiskers on each box represent the range of OD values obtained from each group, median OD values are indicated by the horizontal line through each box, and mean OD values are indicated by the X inside each box.

Screening of RA and NHD serum for *Raoultella*-specific antibodies at a 1:400 serum dilution did not reveal any statistically significant differences in *Raoultella*-specific antibody levels in the two groups (Table 8). The mean OD value of RA serum at a 1:400 dilution was 0.460 greater than NHD serum (t=1.00) when screened against *R. ornitholytica* whole cell lysate. Screening against *R. planticola* whole cell lysate revealed
that the mean OD of RA patient serum was 0.373 higher (t=0.647). When screened against \textit{S. pneumoniae}, the mean OD value of RA serum was 0.172 lower than NHD (t=0.710).

Table 8. Statistical analysis of blank corrected OD values for RA patient and NHD serum samples diluted 1:400 in serum dilution buffer. A Welch’s T-test was used to calculate the T-value and then compared to the critical t-value corresponding to the calculated degrees of freedom.

<table>
<thead>
<tr>
<th>Species</th>
<th>Mean RA serum OD (450 nm)</th>
<th>Mean NHD serum OD (450 nm)</th>
<th>Absolute Difference</th>
<th>T-value</th>
<th>Degrees of Freedom</th>
<th>Critical T-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{R. ornitholytica} (1:400 serum dilution)</td>
<td>1.706</td>
<td>1.246</td>
<td>0.460</td>
<td>-0.708</td>
<td>10</td>
<td>2.23</td>
</tr>
<tr>
<td>\textit{R. planticola} (1:400 serum dilution)</td>
<td>2.240</td>
<td>1.1886</td>
<td>0.374</td>
<td>1.00</td>
<td>9</td>
<td>2.26</td>
</tr>
<tr>
<td>\textit{S. pneumoniae} (1:100 serum dilution)</td>
<td>3.768</td>
<td>3.876</td>
<td>0.108</td>
<td>0.647</td>
<td>9</td>
<td>2.26</td>
</tr>
</tbody>
</table>

**DISCUSSION**

**Establishment of Pure Bacterial Culture**

Per ATCC culturing recommendations, \textit{R. planticola} was cultured at 26°C and \textit{R. ornitholytica} was cultured at 37°C. Repeating this experiment using whole cell lysate from \textit{R. ornitholytica} and \textit{R. planticola} cultured at both 32°C and 37°C is warranted, as such experiment would provide insight into whether or not thermal regulation of
virulence genes impacts differences in *Raoultella*-specific antibodies between RA patients and NHDs.

The core temperature of humans is approximately 37°C, which is typically higher than environmental temperatures. When pathogenic bacteria approach 37°C, expression of virulence factors are often upregulated to promote survival and proliferation within their host (76). Intraarticular temperature of joints is typically lower than core temperature. At room temperature, intraarticular knee temperature is approximately 31.4°C. During synovitis and other inflammatory events, intraarticular temperature approaches core temperature (77).

It is possible that the culturing temperature of each organism resulted in a protein expression profile that is different than what would be observed during a natural infection, synovial translocation, or synovitis. Differences in expression profile due to culturing conditions has the potential to impact measured OD values for human serum, as individuals might have immune responses directed toward proteins that are not expressed at the culturing conditions used in this experiment.

Broth culture population density also has the potential to impact expression of virulence factors of bacteria (78). Broth cultures were not grown to a specific turbidity before they were used to make whole cell lysate. Additional experiments growing broth cultures to specific turbidities prior to generating bacterial whole cell lysate would provide insight into whether or not regulation of virulence genes at different population densities impacts species-specific antibody differences between RA patients and NHD.
ELISA Optimization

ChonBlock™, a mammalian protein-based ELISA blocking buffer effectively reduced background noise and off-target immunoglobulin reactions, as shown in table 7. Negative controls for RA and NHD donors had OD values less than 0.03. In future experiments, a control well that is coated in whole cell bacterial lysate and blocked, but not incubated with serum or secondary antibody will be included. This control would determine the OD of lysate and block in the absence of antibodies. Future experiments should investigate how these results compare to other blocking systems.

Screening RA and NHD Serum

Due to the associative nature of this study, the implications of this data must be considered carefully. Although results are statistically insignificant, it is possible that the elevated OD values observed in RA patients are reflective of a clinical link between RA and *Raoultella*. On the contrary, it is possible that these findings are strictly coincidental or reflective of other intrinsic immunologic differences between RA patients and NHD. Future experiments are needed to further investigate the possibility of this association.

Future work will require verifying that antibody binding in this ELISA is species specific. Elevated endotoxin-specific IgM and IgA antibody titers have been observed in RA patients compared to NHD (79). Therefore, it is possible that the elevated OD values observed in RA patients when screened against *R. ornitholytica* and *R. planticola* is a result of a higher anti-Gram-negative bacterial response, not a *Raoultella* species-specific response. To address this, identifying immunogenic peptides that are unique to *Raoultella*, using recombinant technologies to express them, and screening serum against these peptides could rule out the possibility that observed differences are specific-to
Gram negative bacteria, not *Raoultella* specifically. Western blotting could also be used to screen serum against *Raoultella* lysate to determine potential antibody targets that are unique to *Raoultella*.

Due to budgetary and time restrictions, replicates of this experiment were not performed. Repeating this study would allow for assessment of replicability and patterns, which would allow for better statistical analysis. Expanding upon the number of RA and NHD patients tested is also important. Of the 15 samples tested, only one RA patient and two NHD were male. Additional experiments will include expanding upon the number of RA patients and NHD tested, as well as testing patients and NHD from different demographics to account for possible confounding variables such as age, sex, comorbidities, socioeconomic status, nationality, and geographic location of residence.
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