

High Frequency of Extractable Nuclear Autoantibodies in Wheat-Related Disorders

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ABSTRACT

BACKGROUND AND AIMS: There has been broad interest to explore the presence of autoimmunity among wheat-sensitive individuals, but neither the pathogenesis nor the relevance has been established. In this study, we evaluated the frequencies and levels of autoantibodies, which are important biomarkers of autoimmunity, in subjects with wheat-related disorders and controls. Anti-nuclear antibodies (ANA) and the specific ones against extractable nuclear antigens (ENA) were investigated.

METHODS: A total of 713 subjects who showed symptoms related to wheat ingestion were addressed to Vibrant America Clinical Laboratory from December 2015 to November 2017. Serum samples were collected from all subjects and tested with a wheat protein antibody panel (IgG and IgA to 18 proteins at the peptide level) and an autoantibody panel (ANA by immunofluorescence analysis and 10 ENA antibodies). Retrospective analysis was completed using de-identified clinical data and test results.

RESULTS: In the retrospective analysis, 38 (5%) were seropositive in a Celiac Disease panel, 491 (83%) were seropositive in a wheat protein antibody panel “Wheat Zoomer,” and 84 (12%) were seronegative in both panels. Anti-nuclear antibodies were detected in similar portions of the celiac disease subjects (13%), the Wheat Zoomer–positive subjects (12%), and seronegative controls (15%), which is also very close to the reported occurrence of ANA positivity (15%) in the healthy population. The prevalence of anti-ENA was reported to be less than 2% in the general population; however, our study found it to be much higher in the celiac disease subjects (29%) and the wheat-sensitive subjects (27%), compared with a smaller proportion of seronegative controls (19%). The prevalence of anti-histone was especially prominent among the celiac disease subjects (73%) and the Wheat Zoomer–positive subjects (60%).

CONCLUSIONS: High proportions of wheat-related disease subjects carry ENA antibodies that are important specific biomarkers of autoimmunity.

KEYWORDS: Food sensitivity, gluten, wheat, celiac disease, ANA, ENA, autoantibody, systemic autoimmune disease

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Introduction

Wheat is a complex grain, composed of various molecules of carbohydrates, proteins, and fat. For some individuals, wheat ingestion is associated with a cascade of symptoms from gastrointestinal distress such as diarrhea, constipation, bloating, cramping, and altered bowel habits to extraintestinal symptoms, including fatigue, joint pain, depression, and cognitive difficulties.¹ Among a broad spectrum of wheat-related disorders, celiac disease is the best known: a chronic immune-mediated enteropathy in which dietary gluten leads to small bowel inflammation and villous atrophy in genetically susceptible individuals. Celiac disease affects about 1% of adults and children in the United States.² Another emerging wheat-related disease is nonceliac wheat sensitivity (NCWS), which is a condition with both gastrointestinal and nongastrointestinal symptoms related to wheat or gluten intake that occurs in the absence of celiac disease and wheat allergy. Currently there is no well-characterized biomarker or diagnostic test for NCWS, although 1% to 6% of the population

is estimated to be affected by NCWS.³ Aside from gluten, some of the lesser known components of wheat may also play a role.^{4,5}

Over the past few years, celiac disease has been associated with more than a few autoimmune disorders.^{6,7} Remarkably, in patients with autoimmune thyroiditis (Hashimoto’s thyroiditis, Graves’ disease),⁸ Addison’s disease,^{9–11} autoimmune insulin-dependent diabetes mellitus,¹² Sjögren’s syndrome,^{13–15} and autoimmune hepatitis,^{16,17} the frequency of celiac disease is much higher than that in the normal population. However, there is scarce information regarding systemic autoimmune disorders in celiac disease and other types of wheat-related disorders. Antinuclear antibodies (ANA) and the specific ones against extractable nuclear antigens (ENA) have been recognized as diagnostic features of systemic autoimmune disorders (eg, systemic lupus erythematosus [SLE], mixed connective tissue disorder). Carroccio et al reported in a retrospective study that the prevalence of ANA was 24% in the celiac patients and 46% in



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the NCWS patients.¹⁸ The same group also researched a prospective cohort and found ANAs prevalence to be 7.5% in the celiac patients and 28% in the NCWS patients.¹⁸ In another comparative study by Volta et al,¹⁹ ANA was observed in 49% of the patients with celiac disease, reflecting a predominant autoimmune profile, as compared with 37% in the NCWS patients.

In this study, we researched the frequency and level of ANA and 10 ENA antibodies in seropositive subjects in either celiac disease panel or wheat protein antibody panel and seronegative controls. A total of 713 subjects who showed symptoms related to wheat ingestion were addressed to Vibrant America Clinical Laboratory from December 2015 to November 2017. Serum samples were collected from all subjects and tested with a wheat protein antibody panel “Wheat Zoomer” (IgG and IgA to 18 proteins) and an autoantibody panel (ANA by immunofluorescence analysis and 10 ENA antibodies). Retrospective analysis was completed using de-identified clinical data and test results.

Materials and Methods

Study design and population

A total of 713 subjects with wheat-related symptoms were addressed to the Vibrant America Clinical Laboratory for a routine testing by the Celiac Disease Panel, Wheat Zoomer, ANA Panel, and ENA-10 Profile between December 2015 and November 2017. No restricted diet was instructed to the subjects during the study period. Mean age (\pm SD) of the subjects was 48 ± 16 years. The female to male ratio was 2:1 (69% female, 31% male).

To streamline our study, we have defined the subjects into 3 groups:

- Celiac disease subjects—These subjects were seropositive for at least one antibody in the Celiac Disease panel, whereas they might be seropositive for antibodies in the Wheat Zoomer panel as well. These subjects are considered to be celiac disease suspects based on symptoms and serology test (sensitivity 99%, specificity 100%) without biopsy examination.
- Wheat Zoomer-positive subjects—These subjects were seropositive for at least one antibody in the Wheat Zoomer panel, whereas they were seronegative for any antibody in the Celiac Disease panel. These subjects are suspected to have a degree of wheat sensitivity based on symptoms and serology tests (undergoing clinical investigation, no sensitivity and specificity available); however, they did not implement gluten-free diet to confirm an NCWS diagnosis; therefore, irritable bowel syndrome might be included as well.
- Non-wheat-sensitive subjects—These subjects were seronegative for any antibody in both the Wheat Zoomer panel and the Celiac Disease panel.

Celiac Disease panel

The Celiac Disease panel (Vibrant America, LLC, San Carlos, CA, USA) includes anti-transglutaminase 2 IgA and IgG,

anti-deamidated gliadin peptide (DGP) IgA and IgG. Serum specimens were tested with this panel to assist in diagnosis of celiac disease. All diagnosis was established with combined serologic tests on the fluorescent microarray platform which was proven to have 99% sensitivity and 100% specificity.²⁰ The detailed method for generating the peptide arrays and testing process was elaborated in our previous work.²⁰ In general, the immunoassay binding activities between the antibodies in the serum and the attached peptides were scanned by a fluorescence microarray scanner and the data were analyzed to differentiate the substantial levels of binding, which referred to the mean signal binding intensity of the subsequences. The obtained fluorescent binding intensities were then converted to antibody-binding units after normalizing the values for each peptide and compared with an experimentally determined borderline range (0.95–1.05). Any positivity in the Celiac Disease panel indicates a seropositive celiac disease, except when total IgA was low it indicates an IgA-deficiency celiac disease.

Wheat Zoomer

Wheat Zoomer (Vibrant America, LLC) testing is performed at Vibrant America, a Clinical Laboratory Improvement Amendments (CLIA) and College of American Pathologists (CAP)-certified laboratory, and uses ISO-13485-developed technology. Wheat Zoomer is a comprehensive wheat protein antibody panel covering IgG and IgA to 18 proteins listed in Table 1. The Wheat Zoomer uses a microchip array containing a wide range of wheat-derived peptides, offering specific recognitions to IgG and IgA. All the key proteins of wheat are arrayed on the Vibrant Wheat Zoomer chip as overlapping 18-mer peptides covering the entire protein. These chips are then placed on a 96-pillar plate and assayed against samples to determine their reactivity. The detailed method for generating the peptide arrays and testing process is very similar to the ones as reported in our previous work.²⁰ It also provides a total measurement of IgA and IgG, which are often low in people with gluten sensitivity (though the fact that 10% of the general population may have a genetic IgA deficiency may confound the results).

ANA panel

ANA detection was performed with the Vibrant ANA HEp-2 (Vibrant America, LLC), which is a solid-phase bio-chip immunofluorescence assay designed to detect ANA. Samples were incubated with antigen substrate and unreacted antibodies were washed off by a wash solution. The substrate was incubated with specific fluorescent dye-labeled conjugate and then unbound reagent was washed off. Microarray chip reading was performed on a fluorescent microscope scanner, which then transmitted data to the proprietary software program for analysis. When viewing through a fluorescence microscope, autoantibody-positive samples exhibited a bright fluorescence corresponding to areas of the cell or nuclei where autoantibody was bound. The Hamilton Microlab STAR robotic pipetting

Table 1. Protein probes of Wheat Zoomer.

PANEL	PROTEINS
Celiac	Transglutaminase 2, deamidated gliadin peptide (DGP)
Transglutaminase ^{21,22}	Transglutaminase 3, Transglutaminase 6
Wheat Germ ²³	Wheat germ agglutinin
Gliadin ²⁴	α Gliadin, α - β gliadin, γ -gliadin, Ω -gliadin, gluteomorphin, prodynorphin
Glutenin ²⁵	Low-molecular-weight glutenin, high-molecular-weight glutenin
Nongluten protein ²⁶	Serpin, farnins, amylase/protease inhibitors, globulins, purinin

station used VENUS Two software programming for sample and reagent pipetting and management of plate handling, minimizing assay contamination.

The interpretation of the results depended on the pattern observed, the titer of the autoantibody, and the age of the patient.²⁷ A sample was considered ANA negative (ANA-) if specific staining was equal to or less than a negative control (buffer containing preservative and human serum with no IgG ANA). Samples might exhibit various degrees of background staining due to heterophile antibodies or low-level autoantibodies to cytoplasmic constituents such as contractile proteins. A sample was considered ANA positive (ANA+) if any specific staining (homogeneous, centromere, speckled, nucleolar, peripheral) was observed to be greater than the negative control. The elderly, especially women, are prone to develop low-titered autoantibodies in the absence of clinical autoimmune disease. A 1:40 dilution was suggested as a good dilution to screen for ANA.²⁸ Low-titer positive results might occur in apparently healthy persons; therefore, the ANA results were always interpreted considering the patients' total clinical presentation.

ENA-10 profile

A total of 10 anti-ENA antibodies including SSA(Ro), SSB(La), RNP/Sm, Jo-1, Sm, Scl-70, Chromatin, Centromere, Histone, and RNA polymerase III were tested. SSA(Ro), SSB(La), RNP/Sm, and Jo-1 were detected using a solid-phase bio-chip immunofluorescence assay that reports qualitative and semiquantitative results of IgG to SSA(Ro), SSB(La), RNP/Sm, and Jo-1. Patient results were interpreted by comparison with calibrators, controls, and cut-off values. The assessment and interpretation of the results was following the international guideline announced by the European Autoimmunity Standardization Initiative and the International Union of Immunologic Societies/World Health Organization/Arthritis Foundation/Centers for Disease Control and Prevention autoantibody standardizing committee.²⁸ A sample was considered ENA negative if the concentration of the antibody to ENA was equal to or less than the cut-off value. A sample was considered ENA positive when it has at least one autoantibody to ENA at borderline of or more than an index value of 0.95.

The results for Sm, Scl-70, Chromatin, Centromere, Histone, and RNA polymerase III were obtained with a commercially available ELISA (enzyme-linked immunosorbent assay) kit (Inova Diagnostics, Inc., San Diego, CA, USA). A positive result was decided when units value was more than the cut-off value of 20, which is a weak positive result suggested by the ELISA provider company.

Statistical analysis

Clinical data from the de-identified subjects were included in a randomly sorted database that was processed and analyzed using Java for Windows version 1.8.45. Data were expressed as mean \pm SD when the distribution was Gaussian. Multiple logistic regression analysis was performed to evaluate the association between the presence of ANA or ENA and the other clinical variables evaluated. A *P* value of $<.05$ was considered significant.

Results

Patient clinical characteristics

After exclusion of the incomplete clinical data in which some test results were lacking, 713 subjects who showed symptoms related to wheat ingestion and ordered the Celiac Disease Panel, Wheat Zoomer, ANA Panel, and ENA-10 Profile were included in this retrospective study. Table 2 shows the clinical characteristics of the subjects in this study. There was generally a higher volume of wheat-sensitive subjects in this cohort because individuals with more defined diagnosis such as celiac disease were not usually required to complete all 4 tests.

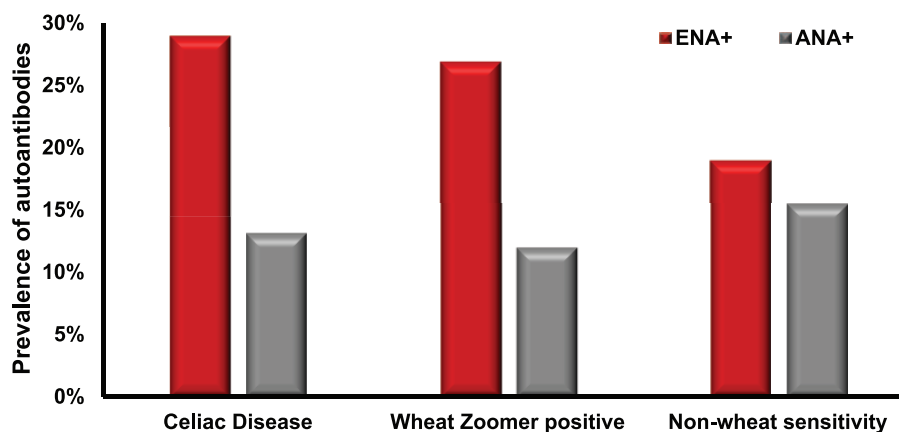
Prevalence and levels of ANA and anti-ENA

As shown in Figure 1, the prevalence of ANA positivity was 13% for the celiac disease subjects, 12% for the Wheat Zoomer-positive subjects, and 15% for the non-wheat-sensitive subjects. The celiac disease subjects, Wheat Zoomer-positive subjects, and non-wheat-sensitive subjects had similar frequencies to be ANA positive (celiac disease, $P=.74$; Wheat Zoomer positive, $P=.40$). Median values of the ANA titer were 1:80 in the celiac disease subjects (range 1:40-1:320),

Table 2. Clinical characteristics of 38 celiac disease subjects, 591 Wheat Zoomer–positive subjects, and 84 non–wheat-sensitive subjects.

	CELIAC DISEASE (N=38)	WHEAT ZOOMER POSITIVE (N=591)	NON-WHEAT SENSITIVITY (N=84)
Age (X±SD)	46±17	48±16	50±16
Sex	24 F/15 M	409 F/182 M	56 F/28 M
ANA+	5/38 (13%)	71/591 (12%)	13/84 (15%)
ENA+	11/38 (29%)	159/591 (27%)	16/84 (19%)
ANA+ and ENA+	1/38 (3%)	3/591 (1%)	1/84 (1%)
ANA– and ENA–	7/38 (18%)	132/591 (22%)	21/84 (25%)

Abbreviations: ANA, antinuclear antibodies; ENA, extractable nuclear antigens.

**Figure 1.** Prevalence of ANA and anti-ENA in subjects with celiac disease, Wheat Zoomer positive, and non-wheat sensitivity. ANA indicates anti-nuclear antibodies; ENA, extractable nuclear antigens.

1:80 in the Wheat Zoomer–positive subjects (range 1:40–1:1280), and 1:80 in the non–wheat-sensitive subjects (range 1:40–1:1280). The ANA pattern was most distributed into “homogeneous” and “speckled,” followed by “nucleolar” and “centromere.” As expected, sex dependence was observed for a female ratio of 90% in the ANA-positive subjects, whereas it was 60% in the initial cohort. Dependence on age was not observed within this cohort.

Subjects with celiac disease and Wheat Zoomer positive were more likely to be ENA positive than patients without wheat sensitivity. The prevalence of ENA antibodies was higher in celiac disease subjects (29%, $P=.04$) and the Wheat Zoomer–positive subjects (27%, $P=.05$), compared with a lower portion subjects without wheat sensitivity (19%).

Frequency of anti-ENA antibodies

As shown in Table 3, anti-Histone was the most frequently found antibody among all anti-ENAs (73% in celiac disease, 60% in Wheat Zoomer positive, and 38% in non-wheat sensitivity). The antibodies against Jo-1, Sm, and RNP were the least prevalent. All 10 anti-ENA antibodies were detected in the Wheat Zoomer–positive subjects. The absence of some anti-ENA in the celiac disease subjects and the non–wheat-sensitive subjects might be due to limited subject sizes.

Prevalence of wheat protein antibodies in ANA-positive or ENA-positive subjects

Among the Wheat Zoomer–positive subjects, the prevalence of wheat protein antibodies was observed in ANA- and ENA-positive subjects, respectively. As shown in Figure 2, antibodies against gliadin and nonglutin proteins were found to have the highest proportions in both ANA-positive (66%, 61%) and ENA-positive (68%, 61%) subjects. The other wheat protein families were less prominent (20%–40%). Antibodies to all the wheat protein listed in the Wheat Zoomer panel were detected with a similar distribution in both groups.

Discussion

Wheat-related disease is an emerging clinical condition which has become more of concern to people due to its high prevalence in general population. A significant number of studies including the present one indicate this to be related with sex, with a much higher frequency in women. Serum biomarkers such as anti-tTG and anti-dGP have been well validated for diagnosing celiac disease; however, the lack of diagnostic markers has remained as a major problem in identifying patients with less defined wheat-sensitive conditions. Even though the copresence of celiac disease with a few other systemic autoimmune diseases was broadly reported, the prevalence of autoantibodies in celiac disease and wheat sensitivity has been underinvestigated.

Table 3. Frequency of anti-ENA in groups.

ENA	CELIAC DISEASE (N=38)		WHEAT ZOOMER POSITIVE (N=591)		NON-WHEAT SENSITIVITY (N=84)	
	FREQUENCY	PERCENTAGE	FREQUENCY	PERCENTAGE	FREQUENCY	PERCENTAGE
Jo-1	0	0	3	2	0	0
Sm	0	0	7	4	0	0
RNP	0	0	1	1	0	0
SSA(Ro)	0	0	14	9	1	6
SSB (La)	1	9	18	11	1	6
Scl-70	0	0	14	9	0	0
Chromatin	1	9	24	15	4	25
Centromere	1	9	17	11	1	6
Histone	8	73	96	60	6	38
RNA polymerase III	1	9	29	18	1	6

Abbreviation: ENA, extractable nuclear antigens.

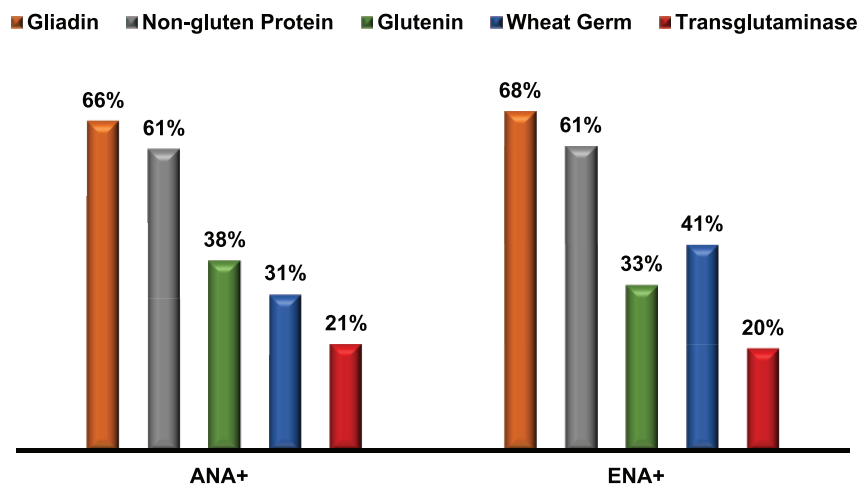


Figure 2. Prevalence of wheat protein antibodies in Wheat Zoomer–positive subjects with ANA positivity or ENA positivity. ANA indicates anti-nuclear antibodies; ENA, extractable nuclear antigens.

In our study, we first researched the prevalence of ANA, a hallmark for diagnosis of systemic autoimmunity, in subjects with celiac disease and in those who were Wheat Zoomer positive. ANA positivity was observed in 5 (13%) of the celiac disease subjects, 71 (12%) of the Wheat Zoomer–positive subjects, and 13 (15%) of the non-wheat sensitivity subjects. Similar comparative studies have been conducted by several other groups and found the results different to some extent. In a study of 56 celiac patients, 118 first-degree relatives, and 101 healthy controls, ANA was detected in 9% of the celiac disease group, whereas ANA positivity was not seen in any of the healthy controls. However, Caglar et al²⁹ reported that there should be no significant difference in the ANA prevalence between these 2 groups, whereas they found it to be 12% (4/31) in the celiac patients and 13.8% (4/29) in the healthy controls. Although our result seems to be consistent with the numbers reported by Caglar et al, we would like to address that ANA positivity exists

in 15% of the apparently healthy population, which is not significantly different from the numbers that we observed.³⁰

According to our own study and some other studies, a subgroup (20%–30%) of the autoimmune patients would have their anti-ENA detected 1–2 years earlier than ANA being detected.^{31,32} In this study, the prevalence of anti-ENA was also observed to be much higher than that of ANA among the subjects. Anti-ENA positivity was observed in 11 (29%) of the celiac disease subjects, 159 (27%) of the Wheat Zoomer–positive subjects, and 16 (19%) of the non-wheat sensitivity subjects. We hypothesize that the prevalence of ANA in this cohort would become more disparate after a longer period of time as a higher proportion of them already carried anti-ENA.

In our study, histone antibody was detected in 73% of the celiac disease subjects and 60% of the Wheat Zoomer–positive subjects. Histone antibody is primarily associated with drug-induced lupus erythematosus, particularly used in distinguishing

drug-induced lupus from other forms of lupus such as SLE, from another autoimmune disorder, or from another cause of a person's symptoms. In a study of association between celiac disease and bacterial transglutaminase in food processing by Lerner et al, core histone was found to be cross-linked by tissue transglutaminase and it could be crucial for chromatin condensation and chromosomal expression.³³ However, there have been no previous studies of histone antibodies in patients with celiac disease or other wheat-related diseases. In all 3 groups evaluated in our study, very few patients (less than 5%) were positive for Jo-1, Sm, and RNP antibody. As far as we are aware, there have been no previous studies of these 3 antibodies in patients with celiac disease or other wheat-related diseases.

Within the cohort of wheat-sensitive subjects, we analyzed the distribution of wheat protein antibody among the ANA-positive and anti-ENA-positive subjects. In both groups, gliadin antibodies were the highest (66% in ANA and 68% in ENA groups), followed by non-gluten proteins (61% in both groups). Although α/γ -gliadin and its deamidated forms are the focus of most commercial tests, the Wheat Zoomer covers all known gliadins from all the different wheat species (α -, γ -, and Ω -gliadins) in both native and deamidated forms. In our study, the high frequency of gliadin antibodies in autoantibody-positive subjects can be contributed to its high prevalence but also the utilization of a more sensitive and comprehensive testing tool. Moreover, nongluten proteins including serpin, farnins, amylase/protease inhibitors, globulins, purinin have been shown to be immune reactive in celiac disease patients.²⁶ In our case, we confirmed the prominent presence of nongluten proteins in other types of wheat-sensitive subjects as well.

In conclusion, our data present a strong tendency toward autoimmunity in patients with wheat-related disorders, characterized by the presence of anti-ENA biomarkers. Therefore, evaluation of autoimmune antibodies is appropriate when existence of an additional autoimmune disease is suspected in patients with wheat-related disorders.

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Author Contributions

YY, HK, KK, and TW performed the research; YY, HK, and VJ designed the study; YY, HK, PD, VR, and KB analyzed the data; YY and HK wrote the article with contribution from all the authors.

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