Age-Related Lectin Histochemical Changes in the Porcine Small Intestine

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ABSTRACT. The carbohydrate histochemistry of normal porcine small intestine from eight one- to four-week-old pigs was investigated by the use of twenty one biotinylated-labeled lectins with avidin-biotin-peroxidase complex method. Five patterns of staining were seen. First, lectins were reacted with brush borders in enterocytes only, i.e. DBA, LEL, STL and PNA. Second, reactivity of lectins were specific for goblet cells, i.e. PSA. Third, lectins were reacted with both brush borders and goblet cells, i.e. RCA I, BSL II, DSL, WGA, Jacalin, ECL, ConA, LCA, UEA-I, PHA-E and PHA-L. Fourth, lectins were positive for brush borders but binding patterns of goblet cells were variable, i.e. BSL I and VVA. Fifth, the binding patterns were variable for brush borders and goblet cells. The binding patterns for SBA, SJA and s-WGA were variable. DSL bound weakly at first-week old pigs, and as the pigs aged, lectin reactivity on the brush borders of enterocytes as well as goblet cells increased. In contrast, STL, PNA and LEL bound strongly at first- and second-week old pigs, and as the pigs aged, lectin reactivity on the brush borders of ileal enterocytes decreased. Our results suggest the distinct age-, region-, and cell types-related changes in epithelial glycocalyx as well as goblet cell mucins in the porcine jejunum and ileum. **KEY WORDS:** enterocyte, goblet cell, histochemistry, lectin, swine.

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Recognition is a central event in a variety of biological phenomenon, such as embryogenesis, cell migration, immune defense and microbial infection [16]. Glycoconjugates comprising the glycocalyx of intestinal epithelium are essential to provide barrier to invading pathogens. The diversity of sugar residues of glycoconjugates is also served as receptor sites for bacterial lectins, thus, mimics or competes with epithelial membrane receptor sites [15]. The exact structure of the carbohydrate side chains of small intestinal mucus glycoproteins is generally difficult since samples contain sloughing cells, bacteria and mucus [14]. This difficulty may be overcome by using lectin since it has very specific binding affinity [8] and is applicable formalin-fixed tissue with histochemical technique [3, 17].

Porcine small intestinal epithelium is the target cells for a variety of enteric pathogens. For example, surface lectins of *Escherichia coli* play a key role in the initiation of infection by mediating bacterial adherence to epithelial cells of the host [16]. In addition, porcine intestinal infections, such as K88 and K99 *E. coli*, are associated with an age relation to infection and disease [18, 19]. Characterization of the carbohydrate profile of the porcine small intestine may be useful in determining whether age-related changes in intestinal surface carbohydrates may be related to susceptibility to infection and/or disease. Age-related surface carbohydrate changes in intestinal cell have been documented in several species, including swine [4, 8, 9, 13]. However, only 7 types of lectin was applied to formalin-fixed intestinal section in swine that may make limitation to define age-related lectin affinity changes for swine intestine. The objective of this study was designed to identify the lectin-binding characteristics of small intestine in pigs of various ages.

MATERIAL AND METHODS

Pigs and sample collection: Small intestinal samples were acquired from 8 conventionally raised Landrace-Duroc cross-bred pigs obtained from a commercial farm. Pigs were euthanatized, using electrocution. Segments of intestines were obtained from middle portion of jejunum and ileum. These small intestines were infused with neutral buffered 10% formalin. After fixation overnight, tissues were processed in paraffin, sectioned at 5 μ m, and mounted on glass slides.

Lectin histochemistry: Tissue sections were stained for lectin binding sites using modification of the avidin-biotin-peroxidase complex (ABC) method [11]. Twenty one lectins were used in this study (Table 1). Following all procedures were performed at room temperature. Serial (5 μ m) sections were deparaffinized in xylene, hydrated through a graded series of alcohols to straight distilled water. Endogenous peroxidase was quenched with absolute methanol containing 0.3% hydrogen peroxide for 15 min. Each section was treated for 1 hr with one of 21 biotinylated lectins (ABC Kit, Vector Laboratories, Burlingame, CA., U.S.A.) at a concentration of 20 μ l/ml in phosphate buffered saline (PBS, pH 7.2). The sections were washed three times with PBS and incubated for 1 hr with an avidin-biotin-peroxidase mixture (ABC, Vector Laboratories, Inc). The sections were washed three times in PBS, and the final reaction product was produced by immersing the sections in a solution of 0.01% hydrogen peroxide and 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in PBS for 15 min. The sections were lightly counterstained with Mayer's hematoxylin, dehydrated through graded concentrations of ethanol and xylene, and mounted. Control tissues were processed without lectin.

Table 1. Lectins used in this study

Lectin/Source*	Abbreviation	Binding specificity
I. N-acetylgalactosamine group:		
<i>Bandeiraea simplicifolia</i> lectin I	BSL I	α -GalNAc, α -Gal
<i>Dolichos biflorus</i> agglutinin	DBA	α -GalNAc
<i>Glycine maxi</i> (soybean agglutinin)	SBA	α -GalNAc
<i>Ricinus communis</i> agglutinin I	RCA I	β -GalNAc, β -Gal
<i>Sophora japonica</i> agglutinin	SJA	β -GalNAc
<i>Vicia villosa</i> agglutinin	VVA	β -GalNAc
II. N-acetylglucosamine group:		
<i>Bandeiraea simplicifolia</i> lectin II	BSL II	α -, β -GlcNAc
<i>Datura stramonium</i> lectin	DSL	β -GlcNAc
<i>Lycopersicon esculentum</i> lectin	LEL	β -GlcNAc
<i>Solanum tuberosum</i> lectin	STL	β -GlcNAc
<i>Triticum vulgare</i> (wheat germ)	WGA	β -GlcNAc > α -NeuAc
Succinylated <i>Triticum vulgare</i>	s-WGA	β -GlcNAc
III. Galactose group:		
<i>Arachis hypogaea</i> (peanut)	PNA	β -Gal
<i>Artocarpus integrifolia</i> (Jacalin)	Jacalin	β -Gal
<i>Erythrina cristagalli</i> lectin	ECL	β -Gal > β -GalNAc
IV. Glucose/mannose group:		
<i>Canavalia ensiformis</i> (concanavalin A)	ConA	α -Man > α -Glc
<i>Pisum sativum</i> agglutinin	PSA	α -Man > α -Glc
<i>Lens culinaris</i> agglutinin	LCA	α -Man > α -Glc
V. Fucose group:		
<i>Ulex europaeus</i> I	UEA-I	α -Fuc
VI. Oligosaccharide group:		
<i>Phaseolus vulgaris</i> agglutinin-E	PHA-E	oligosaccharide
<i>Phaseolus vulgaris</i> agglutinin-L	PHA-L	oligosaccharide

*Lectins are divided into 6 groups depending to their binding specificity.

RESULTS

At the lectin concentration used, labeling was specific and reproducible; non-specific background staining was minimal or absent. Lectin histochemical characteristics of normal jejunal and ileal epithelium from pigs of various ages are summarized in Tables 2 and 3. Heterogeneity of stainability in one cell type was observed even in one section, that is, some cells were positive but others were negative, or some cells were strongly positive but others were weakly. Generally, the lectin tested in this study showed more intense staining in the villus than in the crypt of enterocytes and goblet cells in jejunum and ileum. In addition, all lectins except VVA and SJA had stainability only for brush borders of enterocytes in jejunum and ileum. VVA and SJA showed lectin reactivity for both brush border and cytoplasm of enterocytes.

Jejunal enterocyte: In the lectins of the first group, all lectins except SJA had reactivity for brush borders from all ages of pigs. SJA bound to faintly brush borders from three- and four-week-old pigs, although they share with same binding specificity for beta-N-acetylgalactosamine (Fig. 1). DBA showed a faint staining for the brush borders from all weeks of pigs. BSL I, SBA and RCA I had similar age-dependent reactivity for the brush borders. Namely, the brush borders had gradual decrease in staining for BSL I, SBA and RCA I with increasing pig age. In contrast, the brush borders had gradual increase in staining for VVA

with increasing pig age. In addition, VVA exhibited a moderate diffuse labeling in the brush borders and cytoplasm of villus enterocyte but a diffuse faint staining was seen in the brush borders of crypt.

The lectin binding patterns of the second groups were consistent. All lectins except BSL II and s-WGA had variable reactivity for the brush borders. Only 3-week-old pig failed to have BSL II bound to the brush borders. In addition, one-week-old pigs had BSL II bound to the crypt enterocyte only. Succinylated WGA had strong reactivity for brush border of crypt enterocytes from two-week-old pig but slightly reactivity for the brush border of villus enterocytes (Fig. 2). Brush borders had gradual increase in staining for DSL with increasing pig age. When stained with WGA, the villus enterocytes exhibited more intense staining than the crypt enterocytes (Fig. 3).

All lectins in the third group showed consistent reactivity for the brush borders from all ages of pigs. Brush borders had gradual decrease in staining for PNA (Fig. 4) or ECL with increasing pig age. Jacalin showed an intense diffuse staining for the brush borders from all ages of pigs. In the lectins of the fourth group, ConA and LCA exhibited a diffuse staining for the brush borders, whereas PSA did not show any affinity for the brush borders from all ages of pigs.

In the lectins in the rest of groups, UEA-I and PHA-E and PHA-L showed a diffuse reactivity for the brush borders from all ages of pigs. All ages of pigs had the brush borders

Table 2. Age-dependent lectin binding pattern in the porcine jejunum

Lectin \ Age	1st week E ^{a)} / G ^{b)}	2nd week E / G	3rd week E / G	4th week E / G
Group I				
BSL I	++ ^{c)} / +	+ / -	+ / ++	+ / +
DBA	+ / -	+ / -	+ / -	+ / -
SBA	++ / +	+ / +	+ / -	+ / -
RCA I	++ / ++	++ / ++	+ / ++	+ / ++
SJA	+ / +	+ / -	- / ++	- / -
VVA	+ / -	+ / +	++ / +	++ / +
Group II				
BSL II	- / -	++ / ++	- / -	++ / ++
DSL	++ / +	++ / +	+++ / +++	+++ / +++
LEL	+++ / -	+ / -	+ / -	++ / -
STL	++ / -	++ / -	++ / -	++ / -
WGA	++ / +++	++ / +++	++ / +++	++ / +++
s-WGA	- / -	- / -	- / +	- / -
Group III				
PNA	++ / -	+ / -	+ / -	+ / -
Jacalin	+++ / ++	++ / ++	+++ / +++	+++ / +++
ECL	+++ / +++	++ / ++	++ / +	+ / +++
Group IV				
ConA	++ / +	++ / +	++ / +	++ / +
PSA	- / +	- / +	- / +	- / +
LCA	+ / +	+ / ++	+ / ++	+ / +
Group V				
UEA-I	+ / ++	++ / +++	+ / +	+ / +
Group VI				
PHA-E	+++ / +++	++ / ++	+++ / ++	+++ / +
PHA-L	+ / +	+ / +	+ / +	+ / +

a) Brush border of villus enterocyte.

b) Villus goblet cells.

c) - =negative, + =fair, ++ =moderate, +++ =intense.

that bound to UEA-I, PHA-E and PHA-L; however, there was a significant difference in staining intensity. PHA-E exhibited a intense diffuse staining for the brush borders, while PHA-L showed a faint staining for the brush borders.

Jejunal goblet cells: The lectins of the first group exhibited various staining patterns. Goblet cells from all ages of pigs were stained for RCA I. Goblet cells from one-week-old pig did not react with VVA. None from all ages of pigs had reactivity for DBA. Goblet cells from one- and three-week-old pig were positive for SJA (Fig. 1).

In the lectins of the second group, goblet cells were positive for DSL and WGA. WGA exhibited a intense diffuse staining for goblet cells from all ages of pigs (Fig. 3). Stainability of the goblet cells for DSL had gradually increased with increasing pig age. None from all ages of pigs had reactivity for LEL and STL. The pattern of staining with BSL II was unique and only crypt goblet cells were faintly stained (Fig. 5). Succinylated WGA showed a faint focal staining for goblet cells from three-week-old pig only.

The lectins of the third group, none of the goblet cells from all ages of pigs bound to PNA (Fig. 4), whereas those from all ages of pigs bound to ECL and Jacalin strongly.

Table 3. Age-dependent lectin binding pattern in the porcine ileum

Lectin \ Age	1st week E ^{a)} / G ^{b)}	2nd week E / G	3rd week E / G	4th week E / G
Group I				
BSL I	+ ^{c)} / +	+ / -	+ / +	++ / +
DBA	+ / -	+ / -	+ / +	+ / -
SBA	++ / +	+ / +	- / +	- / +
RCA I	++ / ++	+ / ++	+ / ++	+ / ++
SJA	- / -	+ / -	+ / ++	+ / ++
VVA	+ / -	++ / +	+ / +	++ / ++
Group II				
BSL II	+ / +	+ / +	+ / +	+ / +
DSL	+ / +	++ / +	++ / ++	++ / +
LEL	++ / -	++ / -	+ / -	+ / -
STL	++ / -	++ / -	+ / -	+ / -
s-WGA	+ / -	++ / -	+ / -	- / -
WGA	+ / +++	+ / +++	++ / +++	+++ / +++
Group III				
PNA	+++ / -	++ / -	+ / -	+ / -
Jacalin	++ / +	+++ / ++	+++ / ++	+ / ++
ECL	++ / +++	++ / ++	++ / +	++ / +++
Group IV				
ConA	+ / +	+ / +	++ / +	+++ / +
PSA	- / +	- / ++	- / ++	- / ++
LCA	+ / +	+ / +	- / ++	- / ++
Group V				
UEA-I	+ / +++	++ / +++	+ / +++	++ / +++
Group VI				
PHA-E	++ / ++	++ / ++	++ / ++	++ / ++
PHA-L	++ / +	+ / +	+ / +	++ / +

a) Brush borders of villus enterocyte.

b) Villus goblet cells.

c) - =negative, + =fair, ++ =moderate, +++ =intense.

ConA, PSA and LCA in the fifth group had reactivity for goblet cells from all ages of pigs. UEA-I had reactivity for goblet cells from all ages of pigs. PHA-E and PHA-L of sixth group had similar binding pattern to lectins of the fourth group. PHA-E had slightly higher binding intensity than PHA-L. In sections incubated with PHA-E, a progressive decrease in the stainability of stained goblet cells was observed as the pig aged.

Ileal enterocyte: Generally, the lectins showed more intense staining in the villus than in the crypt enterocytes. In the lectins of first group, BSL I, RCA I and VVA were positive for the brush borders at all ages of pigs (Fig. 6). SBA, RCA I and DBA had reactivity at birth, and as the pigs aged, bound weakly or not at all on brush borders at the three- and four weeks of age. SJA exhibited a moderate diffuse staining in the brush borders and cytoplasm of villus enterocyte. Brush borders of enterocytes only from one-week-old pigs failed to bind to SJA.

All lectins except s-WGA were positive for brush borders from all ages of pigs in the lectins of the second group. Age differences in STL, LEL, DSL and WGA binding were observed. STL and LEL bound strongly to brush borders of

Fig. 1. Jejunum from a 1-week-old piglet. Notice strong lectin affinity for goblet cells (arrow) and fair lectin affinity for brush border of enterocytes. SJA staining, Mayer's hematoxylin counterstain, $\times 200$.

Fig. 2. Jejunum from a 2-week-old piglet. Notice strong lectin affinity for brush border of crypt enterocytes (arrow) and fair lectin affinity for brush border of the villus enterocyte. s-WGA staining, Mayer's hematoxylin counterstain, $\times 200$.

Fig. 3. Jejunum from a 3-week-old piglet. Notice lectin affinity for villus, crypt enterocytes and goblet cells. WGA staining, Mayer's hematoxylin counterstain, $\times 200$.

Fig. 4. Jejunum from a 3-week-old piglet. Notice strong lectin affinity for villus enterocytes (arrow) but lack of lectin affinity for goblet cells. PNA staining, Mayer's hematoxylin counterstain, $\times 200$.

villus and crypt enterocytes at one- and two-week-old and, as the pig aged, lectin stainability decreased. In contrast, DSL and WGA bound to brush borders of villus and crypt enterocytes weakly at one- and two-week-old and, as the pig aged, stainability increased (Fig. 7). The reactivity of the brush borders to s-WGA was lost at four-week-old pigs.

In the lectins of the third group, all lectins were positive to brush borders from all ages of pigs. PNA bound to brush borders of villus and crypt enterocytes strongly at one-week-old and as the pig aged, stainability decreased. In the lectins of the fourth group, the brush borders of enterocyte were

positive for ConA but negative for PSA at all ages of pigs (Fig. 8). Age difference in ConA binding were observed. Stainability of the brush borders to ConA increased as the pig aged. LCA exhibited a faint staining for brush borders from one- and two-week-old pig. The UEA-I bound moderately and diffusely to brush borders in two- and four-week-old pig, while the UEA-I bound faintly and inconsistently to brush borders in one- and three-week-old pig. PHA-E and PHA-L bound to brush borders at all ages of pigs; however, there was a significant difference in staining intensity. PHA-E exhibited a intense diffuse

Fig. 5. Jujunum from a 2-week-old piglet. Notice moderate lectin affinity for cryptic goblet cells as well as brush border of enterocyte (arrow). BSL-II staining, Mayer's hematoxylin counterstain, $\times 200$.

Fig. 6. Ileum from a 4-week-old piglet. Notice strong lectin affinity for cytoplasm of enterocyte (arrow) and moderate lectin affinity for goblet cells. VVA staining, Mayer's hematoxylin counterstain, $\times 200$.

Fig. 7. Ileum from a 1-week-old piglet. Notice strong lectin affinity for goblet cells (arrow) but lack of lectin affinity for crypt and villus enterocytes. WGA staining, Mayer's hematoxylin counterstain, $\times 200$.

Fig. 8. Ileum from a 2-week-old piglet. Notice strong lectin affinity for goblet cells (arrow) but lack of lectin affinity for crypt and villus enterocytes. PSA staining. Mayer's hematoxylin counterstain, $\times 200$.

staining for the brush borders, while PHA-L showed a faint staining for the brush borders.

Ileal goblet cells: In the lectins of the first group, the binding patterns with the lectins were consistent. In the all ages of the pigs examined, goblet cells bound to SBA and RCA I, although staining intensity for RCA I was stronger than that of SBA. In one- and two-week-old pigs, goblets cells were negative for DBA and SJA. Goblets cells were negative for VVA only in one-week-old pigs (Fig. 6).

The binding patterns of lectins in the second group were variable. STL, LEL and s-WGA did not bind to goblet cells

in any age of the pigs examined, whereas BSL II, DSL and WGA bound heavily and diffusely to villus and crypt goblet cells at all ages of pigs (Fig. 7). In the lectins of the third group, goblet cells from all ages of pigs exhibited faintly to moderately diffuse staining for ECL and Jacalin, while none of goblet cells were stained for PNA.

Goblet cells were positive for all lectins tested in the fourth group, ConA, PSA (Fig. 8) and LCA. At all ages of pigs, goblet cells bound to PHA-E and PHA-L; however, there was a significant difference in staining intensity. PHA-E exhibited a intense diffuse staining for the goblet cells,

while PHA-L showed a faint staining for the goblet cells. UEA-I bound intensely to the goblet cells from all ages of pigs.

DISCUSSION

Our studies demonstrate that distinct age-, region- and cell types-related changes in lectin binding to the epithelial glycocalyx as well as goblet cell mucins in the porcine small intestine. Five patterns of staining were seen. First, lectins are reacted with brush borders only, i.e. DBA, LEL, STL and PNA. Second, reactivity of lectins are specific for goblet cells, i.e. PSA. Third, lectins are reacted with both brush borders and goblet cells in jejunum as well as ileum, i.e. RCA I, BSL II, DSL, WGA, Jacalin, ECL, ConA, LCA, UEA-I, PHA-E and PHA-L. Fourth, lectins are positive for brush borders but binding patterns of goblet cells are variable, i.e. BSL I and VVA. Fifth, the binding patterns are variable for brush borders and goblet cells, i.e. SBA, SJA and s-WGA.

Although pathologic processes could result in alterations of intestinal mucins [2], histologic abnormalities were not observed in the intestine of any of the pigs of this study. Generally, villus and crypt enterocyte as well as goblet cell exhibited similar binding patterns for most lectins at various regions of the small intestine with a few exceptions. These regional similarities in goblet cell reactivity indicate a similarities in goblet cells secretion at various regions of the small intestine. Compared with lectins among group, lectins of the second groups showed more staining reactivity than lectins of the first groups. This result indicates that N-acetylglucosamine group express higher affinity to small intestines than N-acetylgalactosamine group. In human being, blood group may influence the glycoproteins that are expressed in the gastrointestinal tract [5]. A and H blood group activities has been detected in porcine small intestinal mucus [13, 14]. A blood grouping lectins such as BSL I, DBA, SBA and VVA have a specificity predominantly for terminal nonreducing N-acetylgalactosamine, while H grouping lectins such as UEA-I have a specificity for alpha-L-fucose. These A and H blood group activities have been reported in porcine small intestinal mucus [13, 14]. Present study supports these findings.

Some lectins showed typical age-related binding patterns. For example, DSL, which have high affinity for N-acetylglucosamine, revealed a typical age-related changes. DSL bound weakly at first-week old pigs and as the pigs aged, increase in lectin reactivity on the brush borders of jejunal and ileal enterocytes as well as jejunal and ileal goblet cells. In contrast, STL, PNA and LEL bound strongly at first- and second-week old pigs and as the pigs aged, decrease in lectin reactivity on the brush borders of ileal enterocytes. It is possible that as the pig aged, the enterocyte acquire affinity for DSL but lose affinity for STL, PNA and LEL. The lectins which have same nominal specificity for carbohydrates showed similar binding patterns. However, the staining pattern of the porcine small intestine by DSL,

STL and PNA was different. We have no explanation for this, but other studies have shown that lectins of the same nominal specificity recognize different features of these complex carbohydrates [6, 12].

Some lectins had cell type-related binding affinity as mentioned above. *Pisum sativum* agglutinin (PSA) did not have appreciable affinity for the brush borders of villus enterocyte. This is in contrast to its affinity for the villus goblet cells. In addition, DBA did not have appreciable affinity for goblet cells, whereas DBA had affinity for the villus enterocyte brush borders. These data were in general agreement with those of a previous study [9]. This observation suggests that the reactivity of the PSA lectin may be primarily due to the terminal alpha-D-glucosyl and alpha-D-mannosyl-like residues on the goblet cells and the reactivity of the DBA lectin may be primarily due to the terminal N-acetyl-alpha-D-galactosaminyl-like residue on the enterocytes.

Crypt enterocyte had similar or slightly lower binding affinity to some lectins than did the corresponding villus enterocyte, for example, BSL I, DBA, SJA and VVA. This indicates the presence of corresponding carbohydrates on the brush borders from early stages of differentiation through maturity of the enterocytes. Unlike above lectins, the reactivity of the BSL II and s-WGA lectins with the enterocytes increased staining with the brush borders indicating loss of N-acetylglucosamine as the enterocyte mature on its way up the intestinal villus.

The reaction of WGA with the luminal surfaces of the enterocytes on the villi is in accordance with other report [8]. In contrast, s-WGA had greater affinity for crypt brush borders than for villus brush borders [9]. The present study shows the increased reactivity of WGA as opposed to s-WGA. Because s-WGA binds terminal N-acetylglucosamine residues and WGA binds terminal N-acetylglucosamine residue, as well as sialic acid residues on the enterocytes, it is possible that as the enterocyte differentiated, cell surface-associated carbohydrate acquire sialylated oligosaccharides.

Lectins that did not have appreciable affinity for goblet cells were PNA, LEL and STL in this study. Selective loss of some PNA receptor, even abolition has been reported to occur in the 10% neutral buffered formalin-fixed and paraffin-embedded tissues [10]. Lack of PNA reactivity to goblet cell mucus in various aged pigs had been also observed in previous study [9]. ConA have not binding to goblet cells in any intestinal region in previous studies [9, 13]. Lack of ConA reactivity to goblet cell mucus in various aged pigs was also observed in this study.

Because our pigs were commercially obtained, we did not have control over environmental or genetic factors that may have influenced the glycoconjugates in the intestines. These have been conflicting descriptions of the binding patterns of lectins. Many of these differences may relate to the diversity of evaluation techniques employed, because a number of variables in methodology, including tissue fixation, sampling regimen, diet and animal availability [1, 4].

Regional and age variations in lectin binding may reflect difference in intestinal function and differentiation. It should be pointed out that terminal residues of intestine may be common to a number of different carbohydrate components, and represented by the reactivity with each lectin. In a view of our results, these differences may be partially responsible for age and regional difference in susceptibility or resistance to enteric disease. More information is needed to define the relationship between age or regional difference and susceptibility or resistance to enteric disease in the future study.

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