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ABSTRACT Poultry production is an important agricultural sector for human food worldwide. Chicks after hatch often face health problems leading to economic losses that are deleterious for breeders. Avian defensin 2 (AvBD2) is a prominent host defense peptide of the intestinal mucosa of cecum and is involved in the resistance of poultry to bacterial pathogens. This peptide could thus represent an innate immunity marker of robustness of birds. To test this hypothesis by comparing fast-growing and slow-growing lines in different conditions of breeding, the chick's cecal AvBD2 content was analyzed according to animal quality and immunity indicators. Chick's cecal tissue sections labeled by immunohistochemistry with newly developed specific antibodies revealed the localization of AvBD2 in the mucosa with high individual variability, without showing differences attributable to quality indicators, but interestingly showing inverse correlation with seric IgM levels in the fast-growing line. The availability of our anti-AvBD2 antibodies to the scientific community opens perspectives to identify the cellular sources of this defensin in the cecal mucosa and to investigate the organization and function of innate immune arsenal of birds.

Key words: chick, intestinal immunity, defensin

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INTRODUCTION

The first days of life are a delicate period for immature chicks developing their digestive and immune capacities after hatch. Chicks are more sensitive to infection by enteric pathogens, which explain why antibiotic treatments and mortality are higher during this period, leading to major losses for breeders and to sanitary risks for humans through the food chain contamination (European Food Safety, A., P. European Centre for Disease, and Control, 2022). The robustness of chicks is so far estimated by rather subjective macroscopic observations such as quality score (Tona et al., 2003). Among innate immunity factors, AvBD2 is a major host defense peptide produced by heterophils and present in cecal tonsils of birds (van Dijk et al., 2023). Its gene expression correlates in the cecum with animal resistance to *Salmonella* carriage (Sadeyen et al., 2006), while transcription level may not always reflect the protein level that is not measurable so far by lack of antibodies specific to the mature peptide of the defensin (36 amino acids; highly folded). Therefore, the development of such antibody could constitute a good tool to monitor this candidate as a biomarker of robustness of chicks. To test this biomarker hypothesis, cecal AvBD2 production measured in cecal tonsils and serum Ig concentration was compared between chicks from different lines and under different breeding conditions with impact on robustness.

MATERIALS AND METHODS

All animal care and experimental procedures were conducted in accordance with current European legislation (EU Directive 2010/63/EU). Animal experiments followed the 3Rs rules (replacement, reduction, refinement) and the ARRIVE guidelines. They were carried out at the INRAE PEAT animal facility (https://doi.org/10.15454/1.5572326250887292E12) registered by the French Ministry of Agriculture (license number C-37-175-1). Fertile eggs were incubated under standard conditions at 37.8°C and 56% relative humidity in the experimental hatchery of PEAT. "Standard" (S) chicks from the fast-growing line Ross 308 and "Label" (L) chicks from the slow-growing line JA657 were subject to environmental challenge after hatch by transport during 30 min which induces shaking, and by

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24 h delayed feeding of chicks maintained at 25°C (conditions close to most of actual breeding practices). Quality of chicks (Tona et al., 2003) was recorded at hatching, at d 1 and at d 7 of age. At 7 d of age, blood collected from ten chicks per line allowed to measure serum concentration of immunoglobulins (IgA, IgM, and IgY) by ELISA using coating (Cat no. A30-103A, A30-102A, and A30-104A) and horseradish peroxidase-conjugated (Cat no. A30-103P, A30-102P, and A30-104P) goat polyclonal anti-chicken IgA, IgM, or IgY antibodies and a chicken reference serum as a standard, all from Bethyl Laboratories (Texas). Animals were euthanized by cervical dislocation before necropsy to collect cecal tonsil tissue placed in paraformaldehyde (4% in PBS) for 24 h and washed three times in PBS. Immunohistochemical (IHC) analysis was performed on serial paraffin-embedded tissue sections (5 μm thickness) on glass slides. Briefly, after paraffin removal, endogenous peroxidase of the intestinal tissue was inhibited and goat serum was applied for 20 min to block potential non-specific binding sites of Ig. Slides were washed twice with PBS, 0.1% Tween 20, and 1% BSA (PTB) between each step. Slides were incubated for 1 h with rabbit anti-AvBD2 polyclonal antibodies (1:2,500 dilution in PTB). These purified anti-AvBD2 IgG were obtained from rabbit antiserum toward natural AvBD2 (available on demand), and were controlled by Western blot and by ELISA as described in a database (doi:10.57745/OYOAWF). Slides were incubated 30 min with secondary antibodies peroxidase-conjugated goat anti-rabbit Ig (Histofine simple stain AP, Nichirei Biosciences, Japan) diluted at 70% in goat serum. Peroxidase substrate (DAB, Labvision, Sweden), giving a brown staining after enzyme reaction, was added for 10 min. Counterstaining was performed with hematoxylin (Papanicolaou reagent, Merck, Germany). After final dehydration in ethanol and Histosol, slides were mounted with coverslips in Eukitt (O. Kindler, Germany). Microscope Eclipse 80i equipped with a camera (Nikon Instruments Europe BV, The Netherlands) was used to observe and record representative images from 10 slides per animal. Images were analyzed with Image J software in order to quantify the percentage of labeled section area.

RESULTS AND DISCUSSION

IHC analysis of AvBD2 in cecal tonsil tissue sections of a representative positive animal from each line is shown in Figure 1. By comparison to negative control using irrelevant rabbit antibodies (Figure 1A and D), specific labeling of AvBD2 was found in the lamina propria and close to crypts of cecal mucosa (Figure 1B and E), in agreement with previous observations (Terada et al., 2018). At high magnification, AvBD2 was found close to capillaries and vessels (Figure 1C and F). These mucosal locations of AvBD2 were observed whatever the line examined and did not appear to correspond to epithelial cells lining the villi. If epithelial cells are known to produce defensins in mammals (O'Neil et al., 1999), the results obtained here show that there is

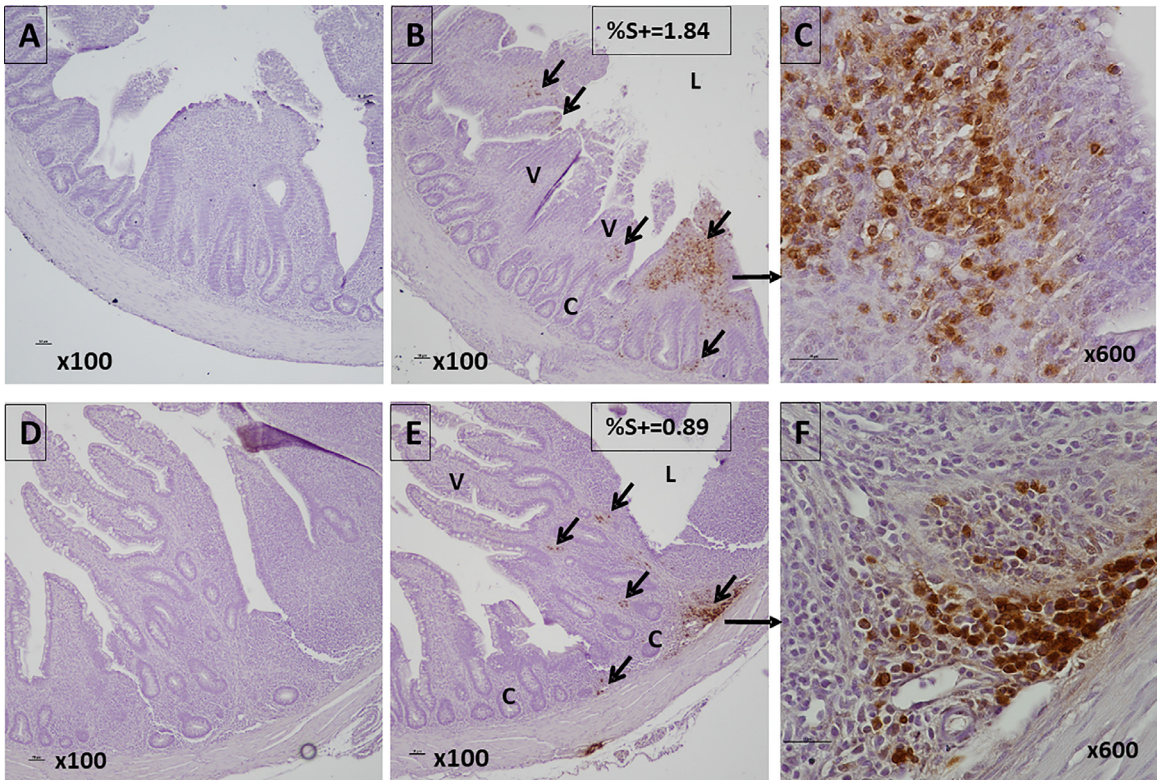

Figure 1. IHC analysis of AvBD2 in cecal tonsil from a representative S chick (top) and from a representative L chick (bottom). Left panels (A, D): normal rabbit Ig used as primary antibody negative control; Right panels (B, C) and (E, F): rabbit anti-AvBD2 polyclonal antibodies; Peroxidase conjugate goat anti-rabbit Ig was used as secondary antibody. Images were analyzed by Image J (Fiji) software to quantify percent area of the tissue section stained for AvBD2 (%S+), as indicated at the top of panels B and E. V: villus; C: crypt; M: muscle; L: lumen.

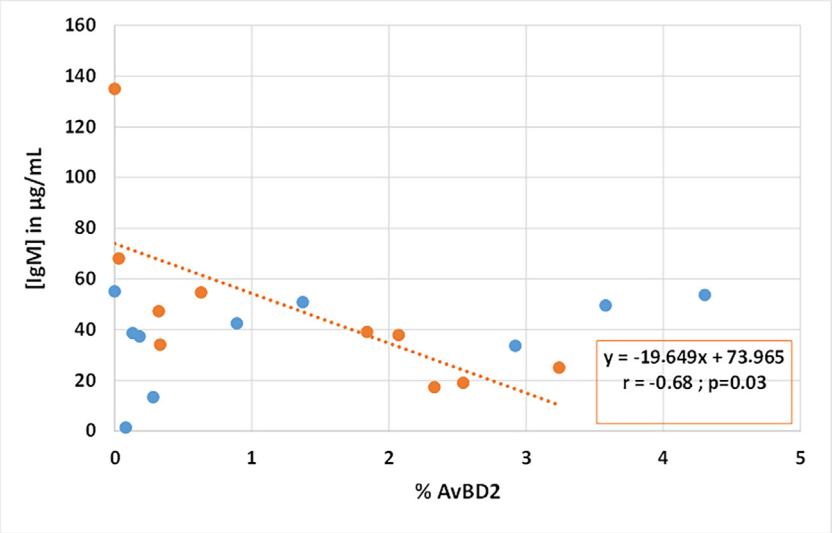

Figure 2. Scatterplot representation of seric IgM concentration as a function of % AvBD2 positive area of cecal tonsil tissue. Values from line L chicks are in blue ($n = 10$) and values from line S chicks are in orange ($n = 10$). For line S chicks, linear regression is in dotted line whose equation as well as Pearson's correlation coefficient r and P values are indicated.

another type of AvBD2 producing cell in the cecal mucosa of chicks. AvBD2-positive areas observed at high magnification (Figure 1C and F) showed the diffuse staining surrounding cells, some of them being highly positive and of large size. This diffusion could correspond to AvBD2 released by producing cells, without being able to affirm the heterophil nature of these cells in absence of available specific antibodies for a probing double staining. The presence of capillaries and vessels at the proximity of AvBD2 producing cells suggests the influx of these cells from the circulation to the mucosa, what is consistent with the capacity of heterophils to infiltrate intestinal mucosa of chicks (Bar-Shira and Friedman, 2006).

The concentrations of serum IgM of 10 chicks from each line were plotted as a function of the values obtained in % AvBD2 positive area of cecal tonsil, as shown in Figure 2. It can be first noticed that there is a high variability in % AvBD2 between individuals for both lines: from 0 to 4% with a mean value of 1.37 ± 1.63% for chicks of line L, and from 0 to 3% with a mean value of 1.33 ± 1.20% for chicks of line S. However, AvBD2 level is not correlated with the quality score value (data not shown; data available in a database at doi:10.57745/OYOAWF). This result indicates this defensin cannot be selected as a potent biomarker of the robustness of chicks. However, it can be observed from the linear regression a significant negative correlation between these immune parameters in the S line chicks. A hypothesis explaining this result relies on an increased immunization by opportunistic bacteria crossing the epithelium of potentially fragilized S chicks, in conjunction with a low mucosal AvBD2 level unable to eliminate bacteria reaching the blood stream. It is known from mammals that intestinal production of defensins may be stimulated by microbiota and/or by inflammatory conditions (Dixon et al., 2016). Keeping in mind that gut microbiota takes place after hatch in chicks, markedly during the first week of life (Lu et al., 2003), future works will be necessary to asses differences in cecal production of AvBD2 between individuals in relationship with microbiota development, and to further characterize producing cells.

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DISCLOSURES

The authors declare they have no competing interests.

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