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Original article

Reliable ELISAs showing differences between resistant and susceptible lines in hens orally inoculated with *Salmonella* Enteritidis

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Abstract – Reliable ELISAs were investigated with the aim to select hen lines resistant to *Salmonella* Enteritidis and producing high levels of antibodies. In the first experiment, the relation between the humoral response and the bacteriological results was assessed on hens from the Y11 resistant line and the L2 susceptible line, orally inoculated with 10^8 CFU *S.* Enteritidis per animal. Anti-lipopolysaccharide (LPS) IgG titres were higher but the liver and spleen were less contaminated in hens from the Y11 line than in hens from the L2 line ($p = 0.013$, 0.031 and 0.026 respectively). In the second experiment, the hens were inoculated orally with 1.7×10^8 CFU *S.* Enteritidis per animal in order to select the ELISA methods showing the more significant differences. ELISAs were based on LPS, flagella, LPS from rough (LPS-R) and smooth strains (LPS-S) and detected IgG and IgM antibodies from sera and yolks. No between-line host response variation was observed in the yolk, with LPS-S and R antigens nor with anti-LPS IgM in the sera. Otherwise, significant differences were encountered between hen lines with the ELISAs performed on the sera detecting anti-LPS IgG, anti-flagella IgG or IgM ($p = 0.017$, 0.017 and $p < 0.001$ respectively). When comparing the kinetics of the selected ELISAs, the IgG antibodies against LPS detected between-line variations as early as 1 to 4 weeks pi, whereas with IgG against flagella, the differences were only detected at 1 and 2 weeks pi and with IgM against flagella, the differences were significant at 1, 2, 4 and 8 weeks pi. In conclusion, resistant hen lines producing higher levels of antibodies than the susceptible hen lines may be selected with these ELISAs.

***Salmonella* / ELISA / resistant and susceptible lines / hen / antibody**

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Résumé – Méthodes ELISA permettant de différencier des lignées de poules résistante et sensible à *Salmonella* Enteritidis inoculées par voie orale. Des méthodes ELISA ont été recherchées pour sélectionner les lignées de poules résistantes à *Salmonella* Enteritidis produisant des taux élevés d'anticorps. Dans la première expérience, la relation entre la réponse sérologique et les résultats bactériologiques a été évaluée sur les poules des lignées résistante Y11 et sensible L2, inoculées par voie orale avec 10^8 UFC *S.* Enteritidis par animal. Les titres en anticorps IgG anti-lipopolysaccharidiques (LPS) étaient supérieurs mais les foies et rates étaient moins contaminés chez les poules de la lignée Y11 que chez les poules de la lignée L2 ($p = 0.013$, 0.031 et 0.026 respectivement). Dans la seconde expérience, les deux lignées de poules ont été inoculées par voie orale avec 1.7×10^8 UFC *S.* Enteritidis par animal afin de sélectionner les méthodes ELISA les plus discriminantes. Les ELISA utilisaient comme antigènes des LPS, flagelles, LPS des souches rough (LPS-R) et smooth (LPS-S), et détectaient des anticorps IgG et IgM dans le sérum et le vitellus. Aucune différence significative n'a été observée à partir du vitellus, ou avec les antigènes LPS-S et R, ainsi qu'avec les IgM anti-LPS dans le sérum. En revanche, les tests ELISA réalisés sur les sérums et basés sur le dépistage des IgG anti-LPS, des IgG ou IgM anti-flagellaires permettent de montrer une différence significative entre les deux lignées ($p = 0.017$, 0.017 et $p < 0.001$ respectivement). La comparaison des cinétiques obtenues avec les tests ELISAs retenus a montré que les IgG anti-LPS permettaient de détecter des différences entre lignées dès 1 semaine jusqu'à 4 semaines après inoculation, alors qu'avec les IgG anti-flagellaires les différences apparaissaient seulement à 1 et 2 semaines, et avec les IgM anti-flagellaires les différences étaient significatives à 1, 2, 4 et 8 semaines après inoculation. En conclusion, les lignées de poules résistantes et produisant des taux d'anticorps plus élevés que les lignées de poules sensibles peuvent être sélectionnées avec ces tests ELISA.

***Salmonella* / ELISA / souches résistantes et sensibles / poule pondeuse / anticorps**

1. INTRODUCTION

Human foodborne salmonellosis remains a significant public health hazard with large direct costs due to human illness and an impact on agriculture and agro-industry, and in particular on the poultry industry. Animals are the major source of *Salmonella* for the food-chain and contamination from the environment is currently a threat to the raising of *Salmonella*-free animals. Selection of lines resistant to *Salmonella* could contribute to a decrease in colonisation, carriage and shedding; consequently animals and consumers would be protected. In this work the serological response was performed on resistant and susceptible lines of hen with the aim to find a discriminant ELISA method allowing us to select resistant lines.

Innate resistance is genetically determined and a few genes are involved in the resistance to *Salmonella* in mice [9]. The first gene *Ity* was mapped to chromosome 1 and many strains of mice, such as BALB/c and C57BL/6, have the susceptible allele [24] which regulates the ability of host macrophages to kill *Salmonella* and other intracellular pathogens [19] or controls the rate of growth of *Salmonella* rather than the rate of killing [4]. Recently Vidal et al. [27] identified *Nramp1* as the gene underlying the *Ity* mutation. The second locus involved in the resistance to *Salmonella* is *Xid*; CBA/N mice with the defective allele are more susceptible to *Salmonella* infection [21]. This gene seems to have an impact on the humoral response and consequently on *Salmonella* infection. The third locus *Toll-like receptor 4 (TLR4)* is the gene determining responsiveness to gram-negative bacterial lipopolysaccharide (*Lps*) which also controls part of the resistance to *Sal*monella [22]. For hens, genetic resistance may be important because they have a natural

general mechanism of resistance against all serotypes of *Salmonella*. The effect on the resistance of the former three candidate genes has been identified as having a role in host resistance [26]: *Nramp1* coding the natural resistance associated macrophage protein 1 [10, 13], *TLR4* the gene underlying LPS [3] and *SAL1* which plays a role in multiplication in the spleen [6]. These genes should be further investigated on the selected resistant or susceptible lines.

Our study was performed on two outbred lines: Y11 a broiler line and L2 a layer type line; the former has been shown to be resistant and the second one susceptible [2, 5, 8, 11, 25]. Caeca from 1-week-old chicks of the L2 line are more contaminated than chicks of the Y11 line after oral inoculation [8] and this presence in the caeca is heritable [5]. Significant differences between hens from Y11 and L2 lines in frequencies of infected ovaries, caeca and spleen but not liver were already observed by Protais et al. [25] after inoculation with *S.* Enteritidis at the peak of laying. Moreover, susceptibility to spleen and caecal contamination are heritable, as shown by Beaumont et al. [2], considering the results four weeks after oral inoculation of 304 laying hens. In our first experiment, these bacteriological results on the liver, spleen, caeca and ovaries were compared to the serological results obtained with an ELISA based on lipopolysaccharides which has been previously evaluated and standardised in France [16, 26].

A better knowledge of the immune response would provide a better characterisation of the resistant animals. Kaiser et al. [15] have shown significant differences between two different broiler lines in antibody response measured 10 days after injection of a *S.* Enteritidis vaccine. However Beaumont et al. [2] have observed a low heritability of antibody response four weeks after the second injection of the *S.* Enteritidis vaccine. Studying the kinetics of antibody response in resistant and susceptible lines could allow to choose a more appropriate post-inoculation interval. The goal of our second experiment was to study the antibody response during the 10 weeks following oral inoculation of *S.* Enteritidis. Moreover according to different countries, different types of antigens are used such as lipopolysaccharides, flagella and fimbrial antigen and various antibodies are considered such as immunoglobulin G (IgG), IgM or IgA in sera or yolks and at mucosal surfaces [1]. Therefore in our study, IgG as well as IgM were considered in both yolk and sera and antibodies against LPS and flagella were measured.

2. MATERIALS AND METHODS

2.1. *Salmonella* strain

The *Salmonella* Enteritidis strain used in this study was PT4, isolated as a human food-borne contaminant, obtained from M. Popoff (Institut Pasteur, Paris). After transfer from stock cultures, the strain was cultivated overnight (15 to 18 h) in brain heart infusion broth (Difco Laboratories, Detroit, USA), incubated at 37 °C. Then, one millilitre from the previous suspension was diluted at a 1:100 ratio in a new brain heart infusion broth, incubated at 37 °C for 18 h.

2.2. Experimental designs

Hens from two outbred lines Y11, L2 and one inbred specific pathogen free (SPF) line were provided by INRA, the HUBBARD-ISA Animal Selection Institute (Lyon, France) and AFSSA, respectively. The Y11 line was selected for meat production whereas the L2 line was selected for egg production.

For each experiment, all animals from one line were hatched on the same day and housed in a common room during the rearing period. Then the Y11 and L2 hen lines were sent to AFSSA just before the laying period. There, the hens were housed in

rooms protected from outside contamination by air filters.

In the first experiment, 6 hens from the Y11 line and 8 hens from the L2 line were housed in the same room and orally inoculated with 10^8 CFU *S*. Enteritidis per animal the same day for both lines. At this date the hens from the Y11 line and L2 line had reached their peak of laying respectively at 29 and 21 weeks of age.

In the second experiment 16 hens from the Y11 line, 15 hens from the L2 line and 12 SPF hens were all kept in individual cages for 10 weeks, so that the sera and yolks could be studied for each hen. Each line was housed in a separate room. Thirty week-old SPF were kept free from *Salmonella* while Y11 and L2 lines were orally inoculated with 1.7×10^8 CFU *S*. Enteritidis per animal the same day. At this date the hens from the Y11 line and L2 line had reached their peak of laying respectively at 28 and 20 weeks of age.

2.3. Samples

2.3.1. First experiment

Sera from each hen were collected before inoculation and at 1 and 2 weeks post-inoculation (pi). Hens were sacrificed 4 weeks pi and the caeca, liver, spleen and ovaries were analysed individually.

2.3.2. Second experiment

Sera from each hen and 3 yolks per hen were collected at 1, 2, 4, 6, 8 and 10 weeks pi. Environmental samples (swabs on cages, feeding device and walls) and faeces were collected 7 days before inoculation and at 1, 2, 4, 6 and 8 weeks pi. Faeces and swabs were sampled by groups of 4 cages and water was distributed by groups of 8 cages. Hens were sacrificed 10 weeks pi and the caeca, liver, spleen and ovaries were analysed individually.

2.4. Bacteriological examination

Samples were diluted in buffered peptone water (Armor Equip. Scientific Lab., Combourg, France) (1:10 dilution w/v) and incubated for 16 to 20 h at 37 °C. The enrichment step used Müller-Kauffmann medium for all the samples incubated at 42 °C for 24 h (Armor Equip. Scientific Lab., Combourg, France) and a modified semi-solid Rappaport- Vassiliadis (MSRV) medium for environmental samples (Biokar Diagnostic, Beauvais, France) incubated for 24 h at 41.5 °C. All Müller-Kauffmann media were isolated in Xylose-Lysine-Tergitol 4 agar (XLT4, Difco Laboratories, Detroit, USA) whereas the MSRV in which migration could be observed were sub-cultured in Rambach agar (Merck, Nogent-sur-Marne, France). Two isolated colonies from each agar were confirmed biochemically and serotyped using appropriate poly- and monovalent typing sera (Sanofi-Diagnostic Pasteur, Marnes-la-Coquette, France).

2.5. Serology

2.5.1. Antigens for the ELISAs

For the first experiment sera were only tested with an ELISA method based on LPS (mixed LPS from smooth and rough strains-Reference L2012, Sigma Chemical Co, St. Louis, USA) obtained by phenol-extraction. For the second experiment, various antigens were investigated: the previous LPS, flagella produced with the Kondoh and Hotani method [17], LPS from the rough strain (LPS-R) and smooth strains (LPS-S) [5, 12].

Briefly, for purification of flagella, 10 mL of Nutrient broth was inoculated with S. Enteritidis strain LA5 and shaken aerobically overnight at 37 °C and was transferred to 1 L of pre-warmed Nutrient broth. The culture was again aerobically shaken overnight at 37 °C before sedimenting

of the bacteria by centrifugation at 8 000 *g* for 10 min. The pellet was resuspended in 0.1 M potassium phosphate buffer pH 6.8 (PPB), blended for three minutes on ice using an Omni 1000 homogeniser (Camlab, Cambridge, UK) and then centrifuged for 20 min at 10 000 *g*. The supernatant was then ultracentrifuged at 50 000 *g* for two hours and the sedimented pellet containing the flagella was reconstituted into 2 mL of 100 mM sodium phosphate buffer, pH 6.8. The concentrated preparation was further purified by gel filtration on a Bio-Sep Sec 4000 HPLC column (Phenomenex, Torrance, USA) and concentrated by ultrafiltration using a centrifugal concentrator (Amicon, Beverly, USA) with a 10 000 Da cut-off membrane. The flagella preparation was depolymerised by boiling in sample buffer for five minutes before resolving on SDS-PAGE with a 15% separating gel

The LPS from smooth and rough strains were produced using the technique described by Hitchcock and Brown [12] with some modifications. A shaken overnight culture of *S.* Enteritidis at 37 °C in 25 mL of trypton soja broth was centrifuged at 4 000 *g* (20 min) at 20 °C. The pellet was washed 3 times in PBS and resuspended in a minimal volume of lysis buffer (625 mM Tris, 2% w/v Sodium Dodecyl Sulfate). The lysate was heated 10 min in boiling water. For protein digestion, 2 mg of proteinase K were added. The mix was incubated for 3 h in a 55 °C water-bath, and at room temperature overnight. Then, for LPS extraction, 3 volumes of acetone were added. The mix was incubated for 24 h at 4 °C and it was centrifuged at 12 000 *g* (20 min) at 4 °C. The pellet was suspended in ultra-pure water, 3 volumes of acetone were added, and this suspension was incubated for 24 h at 4 °C. The mix was centrifuged at 12 000 *g* (20 min) at 4 °C. The pellet containing the LPS was suspended in ultra-pure water, lyophilised, and suspended (1 mg/mL) in PBS. Purity was shown by SDS-PAGE (acrylamide gel 12%). The LPS was visualised by a silver stain [7], and the proteins were stained with Coomassie blue R-250 stain.

2.5.2. ELISA development (Tabs. I and II)

ELISAs based on *S.* Enteritidis antigens as LPS, flagella, LPS-R and LPS-S were derived from the reference method used in France which is based on LPS from *S.* Enteritidis and LPS from *S.* Typhimurium [16]. IgG and IgM antibodies were detected from sera, whereas only IgG antibodies were detected from yolks. Yolks were first diluted at a 1: 10 ratio in PBS.

For IgG detection, an alkaline phosphatase-conjugated rabbit anti- chicken IgG (Reference A-9171, Sigma Chemical Co, St. Louis, USA) was used and the substrate was p-Nitrophenyl Phosphate (Reference 104-105, Sigma Chemical Co, St. Louis, USA). The samples, conjugate and substrate were diluted as shown in Table I.

An additional step was required for the detection of IgM antibodies by ELISA since initially anti-chicken IgM prepared in goat were added (Reference 4608, Nordic, Tilburg, the Netherlands) followed by alkaline phosphatase conjugated anti-goat IgG sera prepared in rabbits (Reference Rockland 605-4502, Tebu, Le Perray-en-Yvelines, France). Samples and reagents were diluted as shown in Table II.

The colour reaction was read with a Dynatech MR 5000 spectrophotometer at 405 and 490 nm. A calibrated optical density (COD) was then calculated to eliminate the background with a negative control sera (OD sample – ODN) / (ODP – ODN) with ODN and ODP meaning OD for the negative control and the positive control respectively.

2.6. Statistical analysis

In each experiment, hens were spread randomly in the room and the cage effect

Table I. Dilutions and doses for methods detecting *S.* Enteritidis IgG antibodies on sera and yolk.

Antigens	LPS	Flagella	LPS-S	LPS-R
Antigen (µL/mL)	1.6	0.6	2	2
Sera	1/600	1/1000	1/300	1/100
Yolk (pre-diluted 1:10 in PBS)	1/60	1/100	/	/
Rabbit anti-chicken IgG conjugate	1/1000	1/3000	1/1000	1/500
Substrate (mg/mL)	1	1	1	1

Table II. Dilutions for methods detecting *S.* Enteritidis IgM antibodies on sera.

Antigens	LPS	Flagella
Antigen (µL/mL)	1.6	0.6
Sera	1/80	1/80
Goat anti-chicken IgM	1/200	1/200
Rabbit anti-goat IgG conjugate	1/500	1/500
Substrate (mg/mL)	1	1

was taken into account. Bacteriological results observed on the Y11 and L2 lines were compared with a Fisher exact test.

In the first experiment, serological results observed on Y11 and L2 lines were tested from 1 to 2 weeks pi by analysis of variance (ANOVA test) for repeated and correlated series since the response observed one week depended on the others. Then the ANOVA test was used at 1 and 2 weeks pi separately in order to know exactly when the ELISA was the most discriminant to show between-line differences.

In the second experiment, the mean value of the 3 yolks were calculated at each time point. Then all the serological results observed on the 3 poultry lines were compared from 1 to 10 weeks pi using the ANOVA test to find a discriminant ELISA method that showed variations between lines. When the ANOVA test was giving significant differences between lines on repeated measures the ELISA was further analysed. The ANOVA test was then used to test the ELISA results obtained each week in order to know exactly when the between-line differences occurred.

3. RESULTS

3.1. Bacteriological results

3.1.1. First experiment (Tab. III)

At 4 weeks pi, contamination in the liver and spleen was significantly higher in the L2 line than in the Y11 line ($p = 0.031$ and 0.026 respectively), whereas the results were not significantly different in the caeca and ovaries.

Table III. Bacteriological results (positive number) on organs at 4 weeks post-inoculation (first experiment).

Lines	*n*	Caeca	Liver	Spleen	Ovary
Y11	6	2	0	1	0
L2	8	6	5	7	1
Statistical significance		NS	$p = 0.031$	$p = 0.026$	NS

Table IV. Bacteriological results (positive number) on environmental samples (second experiment).

	Weeks pi														
	+1			+2			+4			+6			+8		
Lines	Y11	L2	SPF	Y11	L2	SPF	Y11	L2	SPF	Y11	L2	SPF	Y11	L2	SPF
Cages (4)[a]	1	0	0	1	2	0	1	0	0	0	0	0	0	0	0
Feeding device (4)	4	1	0	2	1	0	0	1	0	0	0	0	0	0	0
Walls (4)	4	3	0	1	1	0	1	0	0	0	0	0	0	0	0
Water (2)	2	2	0	1	0	0	1	0	0	0	0	0	0	0	0
Feed (4)	3	4	0	3	3	0	3	0	0	1	1	0	0	0	0
Faeces (4)	4	4	0	4	4	0	3	1	0	0	0	0	0	0	0
Total (22)	18	14	0	12	11	0	9	2	0	1	1	0	0	0	0

[a] Number of samples weekly tested for each line.

Table V. Bacteriological results (positive number) on organs at 10 weeks post-inoculation (second experiment).

Lines	*n*	Caeca	Liver	Spleen	Ovary
Y11	16	1	2	1	0
L2	15	2	0	0	2
Statistical significance		NS	NS	NS	NS

3.1.2. Second experiment (Tabs. IV and V)

The bacteriological results of environmental samples and faeces were all negative 1 week before inoculation. *Salmonella* was eliminated in the faeces from the Y11 and L2 lines until 4 weeks pi. The spread of *Salmonella* was observed throughout the rooms from 1 to 6 weeks pi since feed was still contaminated at 6 weeks pi (Tab. IV). At 10 weeks of age, the 12 SPF hens were free of *Salmonella* while a few hens belonging to the Y11 and L2 lines were still contaminated in the caeca, liver, spleen and ovaries (Tab. V). Four hens from the Y11 line out of 16 had at least one bacteriologically positive organ whereas 2 hens from the L2 line out of 15 were positive. All these

bacteriological results were not significantly different for the hens from the Y11 and L2 lines (Tab. V).

3.2. Serology

3.2.1. First experiment (Fig. 1)

Serological response with the ELISA method based on LPS was significantly higher in sera from the Y11 line than in sera from the L2 line from 1 to 2 weeks pi (p = 0.013) and at 1 and 2 weeks pi (p = 0.006 and 0.011 respectively).

3.2.2. Second experiment (Figs. 2, 3 and 4)

The serological response was low and not significantly different from 0 in the uninoculated SPF hens. No significant difference between hen lines was observed with the ELISA methods on the yolks, with IgG against LPS-R, LPS-S, or IgM against LPS on the sera. However IgG against LPS, IgG against flagella and IgM against flagella were significantly higher in the Y11 line than in the L2 line when all the values were considered from 1 to 10 weeks of age (p = 0.017, 0.017 and $p < 0.001$ respectively). Moreover significant differences between lines were observed at 1, 2 and 4 weeks pi with IgG against LPS (p = 0.005, 0.001 and 0.020 respectively, Fig. 2), at 1 and 2 weeks pi with IgG against flagella ($p < 0.001$ at each date, Fig. 3), at 1, 2, 4 and 8 weeks pi with IgM against flagella ($p < 0.001$, $p < 0.001$, p = 0.003 and 0.009 respectively, Fig. 4).

4. DISCUSSION

Our work shows a relationship between serological and bacteriological results since hens from the Y11 line had high serological response and low spleen and caecal contamination in comparison with hens from the L2 line. Thus serology will be helpful to show between-line variations in further studies.

Therefore, in the first experiment at 4 weeks pi the liver and spleen from the L2 line were significantly more contaminated than those from the Y11 line. Protais et al. [25] also observed significant differences in frequencies of infected spleen between the Y11 and L2 lines of hens. However, this was not the case for the liver after inoculation with *S.* Enteritidis at the peak of laying. As observed by Beaumont et al. [2], further studies are needed to compare liver contamination in resistant and susceptible lines. In our second experiment at 10 weeks pi, concerning the bacteriological results, *Salmonella* appeared to be almost completely eliminated from the organs by the host. Moreover the challenge strain was eliminated in the faeces from both lines no longer than 4 weeks pi. Consequently, it was not possible to observe significant

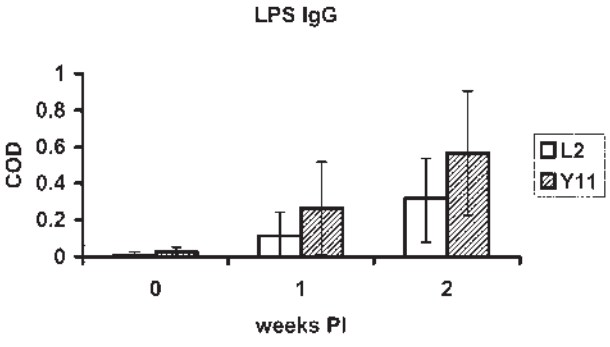

Figure 1. Comparison between Y11 (resistant) and L2 (susceptible) lines with an ELISA test detecting anti-LPS IgG (first experiment).

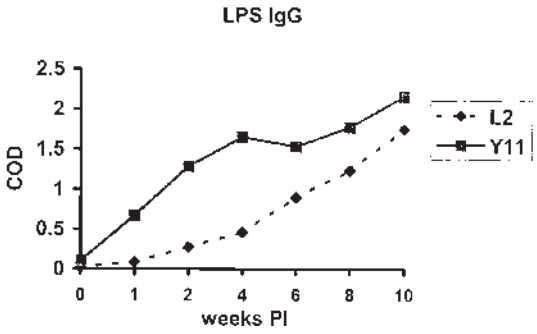

Figure 2. Comparison between Y11 (resistant) and L2 (susceptible) lines with an ELISA test detecting anti-LPS IgG (second experiment).

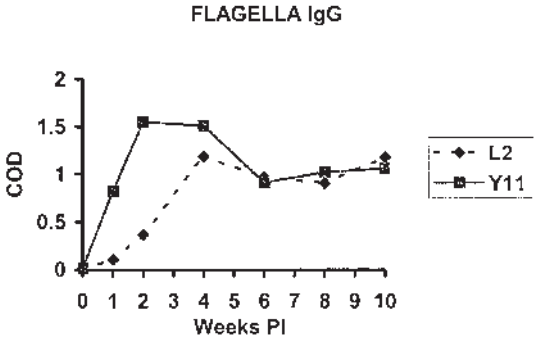

Figure 3. Comparison between Y11 (resistant) and L2 (susceptible) lines with an ELISA test detecting anti-flagella IgG (second experiment).

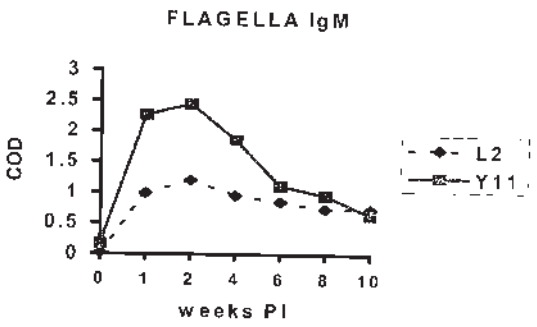

Figure 4. Comparison between Y11 (resistant) and L2 (susceptible) lines with an ELISA test detecting anti-flagella IgM (second experiment).

variations related to the resistance or susceptibility to *Salmonella* Enteritidis. Significant differences have been observed for bacteriological results in previous studies [5, 8, 11, 25] since birds were killed earlier at 4 weeks pi, when *Salmonella* was not eliminated. The aim of our second experiment was different since we were interested in the serological response which occurred later than the bacteriological response.

The IgG production with yolks was not significantly different for the Y11 and L2 lines, although the Y11 line had more IgG with sera; all the antibodies present in the sera were not totally transmitted through the ovaries to the progeny. Locken et al. [20] ascertained that IgG transfer to the ovarian follicles (and thus into the egg yolk) was receptor dependent and that the ovarian IgG receptor allowed the selective transport

of IgG subpopulations presented by the maternal blood. Therefore it appears that the transmission was limited in our study, perhaps because the receptors were saturated or because the transport was selective. Consequently immunoglobulin screening on yolks is not suitable to show variations between lines.

In our study, since we followed IgG or IgM antibody kinetics against LPS or flagellar antigens on sera or yolks it was possible to establish which ELISA was reliable to select susceptible and resistant lines and more importantly, when to use them. Significant variations were observed for serological responses in Y11 and L2 lines in anti-LPS IgG, in anti-flagella IgG and in anti-flagella IgM in sera. Moreover as observed in our experiment, anti-flagella IgG were only reliable to show differences between poultry lines after a recent infection at 1 and 2 weeks pi, whereas anti-LPS IgG were helpful to differentiate the lines from 1 to 4 weeks pi. These results confirmed those obtained previously by Kaiser et al. [15] for IgG against LPS: highly significant differences between poultry lines were observed when they measured antibody responses 10 days after inoculation with a *S.* Enteritidis vaccine. It appears that 4 week pi serological results with anti-LPS IgG are more confusing to find between-line differences. Lastly, significant differences between lines were also observed with IgM against flagella as early as 1 week pi until 4 weeks pi and at 8 weeks pi.

Now, further experiments are needed on antibody response heritability as conflicting results were obtained concerning this subject [2, 14, 18, 23]. In conclusion, in our further studies ELISA would be used on hens to select resistant lines since the present work suggested that resistant lines produced more IgG and IgM antibodies in sera than the susceptible lines of hens.

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