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Published in:
Avian Pathology

Link to article, DOI:
[10.1080/03079457.2016.1267857](https://doi.org/10.1080/03079457.2016.1267857)

Publication date:
2017

Document Version
Peer reviewed version

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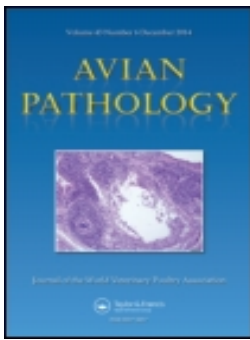
Citation (APA):
Li, L., Thøfner, I., Christensen, J. P., Ronco, T., Pedersen, K., & Olsen, R. H. (2017). Evaluation of the efficacy of an autogenous *Escherichia coli* vaccine in broiler breeders. *Avian Pathology*, 46(3), 300-308.
<https://doi.org/10.1080/03079457.2016.1267857>

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To cite this article: Lili Li, Ida Thøfner, Jens Peter Christensen, Troels Ronco, Karl Pedersen & Rikke H. Olsen (2016): Evaluation of the efficacy of an autogenous Escherichia coli vaccine in broiler breeders, Avian Pathology, DOI: [10.1080/03079457.2016.1267857](https://doi.org/10.1080/03079457.2016.1267857)

To link to this article: <http://dx.doi.org/10.1080/03079457.2016.1267857>



Accepted author version posted online: 16 Dec 2016.



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Publisher: Taylor & Francis & Houghton Trust Ltd

Journal: *Avian Pathology*

DOI: 10.1080/03079457.2016.1267857

Evaluation of the efficacy of an autogenous *Escherichia coli* vaccine in broiler breeders

Short title: Autogenous vaccine experiential trail

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Abstract

In poultry production *Escherichia coli* autogenous vaccines are often used. However, the efficacy of autogenous *E. coli* vaccinations has not been evaluated experimentally in chickens after start of lay. The aim of the present study was to evaluate the protective effect of an autogenous *E. coli* vaccine in broiler breeders. Three groups of 28 weeks old broiler breeders (unvaccinated, vaccinated once and twice, respectively) were challenged with a homologous *E. coli* strain (same strain as included in the vaccine) or a heterologous challenge strain in an experimental ascending model. The clinical outcome was most pronounced in the unvaccinated group; however, the vast majority of chickens in the vaccinated groups had severe pathological manifestations similar to findings in the unvaccinated group after challenge with a homologous as well as a heterologous *E. coli* strain. Although significant titer rises in IgY antibodies were observed in the twice vaccinated group, antibodies did

not confer significant protection in terms of pathological impact. Neither could transfer of maternal derived antibodies to offspring be demonstrated.

In conclusion, with the use of the present model for ascending infection, significant protection of an autogenous *E. coli* vaccine against neither a homologous nor a heterologous *E. coli* challenge could not be documented.

Keywords: APEC, Vaccine, autogenous, challenge study, broiler breeders

Introduction

Avian pathogenic *E. coli* (APEC) is one of the most important opportunist pathogens in industrialized poultry production. APEC is associated with a variety of extraintestinal disease syndromes. In young chicks, APEC-induced omphalitis/yolk sac infection may result in generalized septicaemia with perihepatitis, whereas respiratory infection may occur after aerogenic transmission (Antao et al., 2008; Pires dos Santos et al., 2013). In adult, egg-laying birds, peritonitis and salpingitis are among the most common manifestations of APEC infections, and assumed to occur by ascending bacteria entering the reproductive tract as faecal contaminants through the cloaca (Jordan et al., 2005; Landman and Cornelissen 2006; Pires dos Santos et al., 2013).

Due to the increased concern for antibiotic resistant bacteria, including e.g. extended spectrum beta-lactamase (ESBL) producing *E. coli* (2014), and animal welfare issues, prophylaxis is favoured over antibiotic treatment of *E. coli* infections. It has previously been assumed that disease due to *E. coli* is a result of either management deficiencies, e.g. an inappropriate biosecurity level and insufficient egg hygiene or decreased immunocompetency of the birds due to concurrent infectious diseases, such as infectious bronchitis or adenovirus (Koncicki et al., 2012; Matthijs et al., 2003; Peighambari et al., 2002). Currently, accumulating evidence has led to the acceptance that certain

clones of *E. coli* may act as primary pathogens and cause severe disease and high mortality despite of high standard management, low stress levels and the absence of concurrent diseases (Bisgaard et al., 2010; Dziva et al., 2013; Gregersen et al., 2010; Pires dos Santos et al., 2013). In such cases, control of *E. coli* infections relies on efficient vaccination strategies. The licensed vaccine, Poulvac® is indicated for protection in young birds (broilers or chicks to become broiler breeders or layers) against septicaemia, peritonitis and perihepatitis, and has proven effective in several experimental evaluations (Fernandes et al., 2013; Mombarg et al., 2014; Sadeyen et al., 2015b). For Poulvac®, the therapeutic indications do not include long-term protection beyond the 5-6 weeks of the life of broilers. Field observations concerning *E. coli* mortality in Poulvac® vaccinated/unvaccinated broiler breeders after start of lay (personal communication, poultry field veterinarian), has called for further vaccine-based long-term protection of broiler breeders. Consequently, autogenous vaccines are frequently used in broiler breeder flocks, despite documentation on the efficacy of such vaccines in terms of ascending *E. coli* infections is lacking. Therefore, the aim of this study was to investigate the additive protective effect of an autogenous *E. coli* vaccine in broiler breeders pre-vaccinated with a licensed *E. coli* vaccine (Poulvac®). The purpose was to assess the level of protection against a homologous and a heterologous *E. coli* strain following experimental infection. In addition, transfer of maternal derived antibodies (MAD) was assessed by comparing MAD levels in offspring of vaccinated breeders with offspring of unvaccinated parents.

Material and Methods

Experimental groups and housing

Sixty broiler breeders (genetic line Ross 308) were purchased from a commercial breeder, where they were housed until 27 weeks of age. As day-old and at 12 week of age, all broiler breeders had

been vaccinated with a licensed, live, attenuated *E. coli* vaccine (Poulvac® *E. coli*), in which all birds of all groups had been vaccinated with a coarse spray of Poulvac® according to the manufacturer's instructions. At 28 weeks of age birds were subsequently divided into three experimental groups of 20 chickens per group. Chickens in group one did not receive further *E. coli* vaccines. Chickens in group two were at 18 weeks of age vaccinated subcutaneously with a formalin inactivated, mineral-oil adjuvant *E. coli* vaccine containing three isolates of *E. coli* (an *E. coli* isolate S117, serotype O78:H4 (*E. coli* E44), obtained from the liver of a diseased broiler breeder in 2015 (Ronco et al., 2016), another isolate of *E. coli* ST117 (serotype unknown) obtained from a cellulitis lesion in a broiler in 2015 and finally, an isolate of *E. coli* ST140, serotype O2:H5 (*E. coli* E51), obtained in 2015 from the liver of broilers suffering from septicaemia (Ronco et al., 2016). Chickens in group three received the autogenous vaccine twice, namely at 14 and 18 weeks of age.

At 27 weeks of age all birds of the three groups were transferred to the experimental housing unit at University of Copenhagen. They were allowed one week of acclimatisation before the experimental infections. During the acclimatisation week the birds were monitored for wellbeing, normal avian behaviour and appetite. Birds were kept free range on deep litter in separate floor pens (2.4 × 2.4 m) with unlimited access to nests, perches and dust baths. Water was provided *ad libitum* in all groups throughout the experiment. The birds were kept on restricted diet according to age and egg production (170 g/hen/day). All procedures performed on the birds were approved and licensed by the Danish Animal Experiments Inspectorate (license no. 2013-15-2934-00923).

Preparation of inocula

All strains were stored at - 80°C in Brain and Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) in 15% (v/v) glycerol until needed. The day before inoculation all strains were grown overnight in

BHI broth. The inoculum for each strain was diluted to 5×10^6 CFU, and 0.1 ml of the diluted inocula were aspirated in 1ml syringes and kept on ice until infection.

Oviduct infections and post mortem assessment

The study setup was designed to mimic field conditions. The birds were divided into three groups, each containing 20 birds. Within each group, two subgroups, A and B, were established (Table 1). For each group, subgroup A consisted of birds 1-10 and were infected with the vaccine strain *E. coli* E44, while birds in subgroup B (birds 11-20) were infected with *E. coli* SCI_07 (serotype O non-typable:H31). The latter strain is a Brazilian isolate obtained from periorbital skin of a chicken suffering from "swollen head" syndrome (Rojas et al., 2012), and assumed to represent heterologous *E. coli* strain. The experiments were performed as described by Pors et al.(2014). Briefly, each hen underwent laparotomy under general anaesthesia and received a dose of 0.1ml of approximately 5×10^6 CFU injected into the salpinx. To avoid post-surgical pain a semisynthetic opioid, buprenorphine (0.3 mg/kg), was administered to all hens in all experimental groups. Additional buprenorphine treatments was administered individually if signs of discomfort and due the infection (ruffled feathers, clumping together, reduced appetite and activity), if these signs persisted for more than 12 hours despite treatment or if the symptoms were severe the birds were euthanized for welfare reasons, according to the predefined humane end points in the licence approved by the animal expectorate. Unless indicated by clinical symptoms and significant abnormal behaviour, all chickens were euthanized seven days post infection (d.p.i.) and directly submitted to *post mortem* analysis , where lesions were scored according to the scorings system outlined by Pors et al. (2014). Briefly, each organsystem (ovary, peritoneum and salpinx) was evaluated based pre-defined criteria and assigned a score between 0 (no macroscopically pathology) and 4 for each criteria assessed. The total cumulative score is the sum of scores for all evaluated organsystems in addition to scores on spleen proliferation and lymphatic reaction. Two pathologists

scored all lesions of all chickens during the *post mortem* examination, and if any incongruence in scoring occurred, a third pathologist also participated in the scoring process.

Bacteriological swabs were sampled from liver, bone marrow and salpinx and plated directly on agar plates (Oxoid, Basingstoke, UK) with 5% bovine blood. Re-isolation was considered positive if abundant growth in pure culture of *E. coli* was obtained.

Serology

The antigen response following challenge with *E. coli* E4 and SCI_07, respectively, were determined by Enzyme-Linked Immunosorbent Assay (ELISA). For all birds, blood samples of 5 ml full blood were obtained at the day of challenge and the day of euthanasia (seven days post challenge, except group 1A, which was euthanized 48 h post challenge). The samples were then centrifuged at 4000×g for 10 min. The plasma fraction was transferred to separate tubes and kept at -20 °C until they were processed for ELISA measurement of antibody content. For ELISA, Nunc-Immuno™ MicroWell™ 96-Well Plates (Thermo Scientific, Waltham, MA, USA) were coated overnight at 4 °C with 0.5 µg whole cell sonicates of *E. coli* E4 and SCI_07, respectively. The sonicates were diluted in carbonate-bicarbonate buffer (pH 9.6) (Sigma-Aldrich, St. Louis, MO, USA) to obtain a concentration of 5 mg/ml antigen protein. Each well was then washed; this and all subsequent washing steps consisted of three washes in 350 µL washing buffer (PBS + 0.05% Tween 20). The wells were blocked for 2 h at room temperature in 200 µL blocking solution (PBS containing 0.05% Tween 20 and 2% bovine serum albumin (BSA) and washed. Serum samples from five birds were pooled, and the antibody titers were assayed by serial 3-fold dilutions of serum ranging from 1:300 to 1:24300. All dilutions were prepared in triplicate in dilution buffer (PBS containing 0.05% Tween 20 and 0.1% BSA), 100 µL were added to each well and plates were incubated for 1 h at 37 °C. For each assay, 12 control wells were included, which contained pure dilution buffer; in 6 of these wells a secondary antibody was added as a measure of background, and the other 6 wells remained blank as a negative control for the ELISA. Following incubation, the

wells were washed and 100 µL polyclonal goat anti-chicken IgG (Fc):HRP (AbD Serotec, Puchheim, Germany), diluted 1:4000 in diluting buffer, were added to each well and the plates incubated for a further 1 h at 37 °C and then washed. To detect the binding, 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate (Sigma) were added to each well. The plates were incubated for 2 min and then the reaction was stopped by addition of 100 µL 1 M HCl. The absorbance was read immediately at 450 nm in a PowerWave XS spectrophotometer (BioTek Instruments, Winooski, VT, USA).

The antibody titres were calculated for the measured absorbance at 450 nm (Villumsen and Raida 2013), using the “Antibody Titters” online data analysis tool “Myassay” (2016).

Assessment of maternal derived antibodies in the offspring of autogenously vaccinated and non-vaccinated broiler breeders, respectively, was done as described above, except SCI_07 was replaced by a sonicate from an isolate of the second *E. coli* included vaccine (*E. coli* E51) to verify if any or both of the vaccine strains generated detectable MADs.

Statistical analysis

For the pathology assessment, Kruskal–Wallis followed by Dunn’s Multiple Comparison test was used for comparisons of lesion scores. For detection of differences in lesion scores between challenge strains within the same group (1x autovaccinated), the Mann-Whitney t-test was used for comparing median scores. For serology, two-way ANOVA was used to analyse for statistical significant rise in antibodies in vaccinated groups (2A, 2B, 3A and 3B) compared to the level of antibodies before and after challenge in the control groups (group 1A and 1B). Subsequently, Turkey's multiple comparison test was performed to evaluate if the level of antibodies before and after challenge differed significantly within each challenge group.

Seven days survival rate was compared between the six groups using chi-square tests.

All the statistical analysis was done with Graphpad Prism (Graphpad Software, Inc., La Jolla, USA). P-values <0.05 were considered statistically significant.

Results

Experimental infections

Overall, group 1A (not autogenously vaccinated, challenged with vaccine strain E44) was the only group that had a significantly different survival rate (lower) compared to any of the other groups ($P < 0.001$) (Table 1). Besides mortality, the design of the study included pathology (lesions score) as the main criteria to evaluate the possible protective effect of an autogenous vaccine, while the clinical appearance of the birds at the time from challenge to euthanization was not standardized to be included as criteria for assessment for vaccine efficiency. Nevertheless, based on the animal experiments legislation compulsory, routinely daily monitoring and registration of the wellbeing of the birds, significant differences/changes in the clinical appearance between groups after challenge was observed. The monitoring was carried out at least three times each day; in the morning and afternoon (feeding times) by certified and trained animal caretakers and in the evening the birds were attended by a veterinarian. Initially, no clinical signs were observed in group 3A and 3B, they had normal appetite (all birds were alert during feeding and all feed was eaten) after challenge, they moved around freely in their pens and generally showed normal avian behaviour, including dust-bathing and escape reaction when their pen was entered by a caretaker/veterinarian. However, at day 5-6 post infection, both groups had a decrease in clinical performance and showed signs of being depressed, similar to the signs observed for group 2A and 2B (see below).

The clinical performance for group 2A and 2B indicated that these groups were more severely affected. More birds had ruffled feathers, tended to squeeze into the corners of the pen and some lacked interest in feed.

The clinical appearance of group 1B was similar to the birds in group 2B. For group 1A, three birds died within 24 hour post challenge, while the rest was very severely affected. All birds were anorexic; little or no interest in the surroundings; they had abdominal respiration with beaks open, and tended to group together in the pen. Also, they exhibited weakened escape reaction. For welfare reasons the remaining seven birds were euthanized two days post challenge in line with the predefined humane endpoints.

Overall, at post mortem examination varying degrees of peritonitis, salpingitis and oophoritis was found. For the vast majority of all birds irrespective of group, gross macroscopic lesions were observed, but within groups large variation was demonstrated (Fig. 1). The birds of group 1A had the highest median pathological scores for total lesion, ovary and salpinx (Table 3, Fig. 1).

However, scoring of this group was biased as time of challenge was only two days versus seven days for the remaining groups. None of median cumulative scores of lesions differed significantly between groups ($P < 0.100$), which partly may be explained by the large within-group variation (Fig. 1).

Development of severe diffuse effusive/exudative peritonitis, where the peritoneal membranes had an opaque and thickened appearance, was evident for all groups and peritoneum lesion scores did not differ between groups ($P = 0.594$). Large amounts of fibrinopurulent exudates were present around all organs and spread all over the abdominal cavity.

Inspection of the reproductive organs, ovary and salpinx (Fig. 1C+D) demonstrated that infection of the salpinx was very limited in approximately half of the birds in group 2, regardless of the

challenge strain, corresponding to the lack of *E. coli* re-isolation from these birds (Table 2). Overall the most severe changes in pathology of the salpinx was observed in group 1A, in which the pathology score were significantly higher than the other groups (Fig. 1D) . For both strains the lesions present in the ovaries of all the bird were light to moderate circulatory changes including congestion of blood vessels, multifocal to diffuse dispersion of pus around and within the follicles, and several degenerative and/or regressive follicles. Regarding the oviduct intraluminal presence of purulent exudate ranged from single flakes to diffuse distribution throughout the salpinx. In the mucosal membranes of the salpinx, tissue circulatory changes like oedema, hyperaemia or pale mucosa could be observed within each group. No significant difference in lesion scores of the ovaries was observed between groups ($P=0.231$)

Bacteriology

The rate of bacterial re-isolation is presented in Table 2. Briefly, re-isolation was considered positive, if bacterial swabs from liver, bone marrow or salpinx resulted in pure, fair or florid growth of *E. coli* when cultured on blood agar plates overnight. The highest re-isolation rate of *E. coli* from bone marrow (indicating septicaemia) was found in the in group 1A, in which all birds either had died or were euthanized within 48 h post infection. None of the birds from the remaining groups tested positive from the bone marrow, and except a single bird in group 2A, these birds also cultured *E. coli* negative from the liver (Table 2), indicative of no haematogenous spread present at sampling. All birds in group 1A also tested positive from *E. coli* in the salpinx, all with massive/florid growth of *E. coli* on blood agar, and so did all birds in groups 2B. For the remaining groups, 3 – 6 birds per group *E. coli* could not be isolated by mucosal swabs from the salpinx (Table 2).

Serology

For all samples in all experimental groups, the titre value at the day of challenge ranged from 5,000 to 6,000 (Fig. 2A and 2B). For the groups of chickens challenged with the vaccine strain *E. coli* E44, the relative increase in antibodies against the vaccine strain was highest among birds vaccinated twice, followed by the group of birds vaccinated once. All chickens in group 1A (non-autogenous vaccine; challenged with vaccine strain) were either dead or had to be euthanized 48 hours after challenge, therefore blood had to be obtained already 48 h after challenge (and only included birds that was euthanized) For this group, a four times rise in SCI07-antibodies was observed (Fig 2B), while rise in *E. coli* E44-antibodies were not detected (Fig. 2A).

For birds challenged with *E. coli* E44, only birds which had been autovaccinated twice had a significant rise in antibodies *E. coli* E44-antibodies after challenge ($P < 0.020$). However, un-(auto)-vaccinated birds challenged with *E. coli* E44 had a significant rise of SCI07-reacting antibodies.

For birds challenged with SCI07, birds which had been autovaccinated twice had a significant rise in *E. coli* E44-reacting antibodies after challenge ($P < 0.014$). None of the remaining challenge groups had a significant rise in antibodies reacting towards either *E. coli* E44 or SCI07 (In all cases $P > 0.05$)

For broilers, the level of MAD was not significantly different in broilers originating from vaccinated parents compared with broilers originating from unvaccinated parents (Fig.3), neither with respect to *E. coli* E44 antibodies ($P = 0.094$) or *E. coli* E51 antibodies ($P = 0.3698$)

Discussion

As with all production, cost-benefit of a given action must always be evaluated to determine if the action should be implemented, continued or suspended. Ascending infection due to *E. coli* is the most common infection in broiler breeders and has major impact on production economy (Jordan et al., 2005) Many breeder farms routinely vaccinate broiler breeder chicks with Poulvac®, and although the use of autogenously based *E. coli* vaccines is also widely implemented in industrial poultry production (Deb and Harry 1976; Trampel and Griffith 1997), this study is, to the authors knowledge, the first to evaluate the additive effects of autogenous vaccines in an experimental model for ascending infections.

Judged by the clinical observations of the different experimental groups, an initial assumption of a protective effective of the autogenous vaccine was made, as none of the birds in the non-autogenously vaccinated groups survived the full experimental period when challenged with the vaccine strain *E. coli* E44 (Table 1). Rather, within 48 h after challenge all birds had either died due to infection or been euthanized due to animal welfare reasons. In the groups of birds which had been autogenously vaccinated once or twice, respectively, survival rates were significantly higher when challenged with the autogenous vaccine strain (Table 1). In contrast, survival rates among groups of birds challenged with heterologous *E. coli* were similar between groups independent of the autogenous vaccine status. These initial observations suggest that there is a protective effect of autogenous vaccines, at least evaluated in a seven days trial. However, birds in the groups which had been vaccinated twice seemed merely to have a delay in the onset of clinical disease, as these birds showed increasing symptoms of depression and discomfort during day 5-6 post challenge, e.g. progressing lack of appetite and abdominal respiration among the majority of the birds). These

findings were even more pronounced in the group of birds that had only been autogenously vaccinated once. Moreover, the clinical appearance of birds in the *E. coli* E44 groups on day 5 – 7 post challenge did not differ notably between groups challenged with SCI07, leading to suggest that the autogenous vaccine had low protection against a homologous as well as heterologous *E. coli* challenge under these experimental conditions. Although speculative, based on the clinical appearance it is likely that mortality in all groups would have increased significantly after the seven days post infection, at which the experiment was terminated.

Gross pathology varied markedly within each group (Fig. 1), in accordance with a previous report with the use of the same experimental model, indicating that host-related factors may be of major importance for the outcome of an ascending *E. coli* infection (Olsen et al., 2016). Apart from birds from group 1A, which all had died or had to be euthanized at 48 h, all groups had similar lesions, dominated by severe, fibrinopulent peritonitis, while approximately half of the birds had little indication of salpingitis and also tested bacteriological sterile from this organ. Only birds from group 1A (non-autogenously vaccinated, challenged with vaccine strain *E. coli* E4) sampled *E. coli* positive from the bone marrow, indicating a septicaemic condition at time of death. Whether birds in the other groups might also had an initial state of septicaemia but had been able to clear themselves within the following five days of infection, is only speculative. However, as none of birds in group 1B (non autogenously vaccinated, challenged with SCI07) cultured *E. coli* positive from the bone marrow, a possible bacterial clearness cannot be assigned to the protective effect of the autogenous vaccine, at least not for the heterologous challenge strain.

It may be argued if the infective dose was too high compared to the level of infective bacteria the birds would encounter under field conditions. The infective dose had be chosen based previous

studies (Olsen et al., 2016; Pors et al., 2014) under the assumption that this dose would be high enough for the birds to develop substantial pathology in the non-autogenously vaccinated control group, yet low enough to allow the birds to survive until the planned euthanasia seven days post challenge. In addition, previous studies using the same model have documented that in case the virulence of the strain is sufficient low, an infective dose corresponding to the dose used in the present study, will not result in neither clinical affection nor substantial pathology (Olsen et al., 2016). Assuming the autogenous vaccine would have had a substantial degree of protection, it may be suggested that the outcome of infection in the previous study would have been considerably less severe, even with the use of the current infection dose of 5×10^5 CFU/bird.

Serology results indicate that the vaccine stimulates an IgY antibody response, which significantly increased after a post vaccination challenge of the vaccine strain (Fig. 2) and being most pronounced in the groups autogenously vaccinated twice. However, this antibody response was not specifically related to a post vaccine challenge of the vaccines strain *E. coli* E44, as birds challenged with SCI_07 also had an increase of antibodies directed against the vaccine strain. Hence, it is more likely that strain independent antibodies against *E. coli* are stimulated by the autogenous *E. coli* vaccine (or the Poulvac®) vaccine, and since a whole-cell sonicate was used as antigen in the ELISA assay such antibodies may bind to various epitopes common to different strains of *E. coli*. Adding to the hypothesis that the antibody response is Poulvac® vaccine dependent is supported by the finding that the non-autogenously vaccinated chickens had higher rise in antibodies when challenged with SCI_07 than the autogenously vaccinated groups. The same increase was not observed when the non-autogenously group was challenged with *E. coli* E44, indicating that crossprotection between SCI07 and the Poulvac® strain exists, despite being of different sequence- and serotype. Notwithstanding that the rise in antibodies against *E. coli* E44 was significantly higher in vaccinated than non-vaccinated groups, these antibodies did not confer

significant protection in the present study. The lack of correlation between IgY antibodies and level of protection is not an uncommon observation in studies of avian bacterial infection. In fact, some studies have documented convincing effects of a bacterial vaccine candidate, although no rise in IgY antibodies could be detected (Persson and Bojesen 2015). This may likely be explained by another, antibody independent mode of vaccine protection, e.g. it has been suggested that Poulvac® induced immunity probably does not depend on the production of circulating antibodies (as assessed through the presence of B lymphocytes) but is linked to the presence of CD4⁺TCRVβ1⁺ (Fernandes et al., 2013). This statement, however, conflicts with the finding by Sadeyen et al. (2015b), who showed that both Poulvac® and an inactivated culture of the same strain as included in the Poulvac® vaccine (APEC O78) stimulated a cell-mediated as well as a pronounced humoral response in an intra-air sac model of acute colibacillosis in turkey poults. Yet, another study demonstrated that a protective effect of Poulvac® vaccines strictly requires a cyclophosphamide-sensitive cell population that includes B cells (Sadeyen et al., 2015a).

For a vaccine aiming to protect broilers through vertical transfer of antibodies, high levels of circulating antibodies are crucial. In the present study, the transferability of the antibodies may also be questioned, as day-old broilers of vaccinated parents did not differ in antibody titres from day-old offspring of un-vaccinated broiler breeders (Fig. 3).

In conclusion, vaccination with an autogenous vaccine seemed to stimulate an IgY antibody response when birds were challenged with a homologous strain, but only if the birds had been autogenously vaccinated twice. Based on pathology the autogenously vaccine did not confer protection against neither a homologue nor a heterologous challenge strain in the applied experimental model. Nevertheless, the autogenous vaccine seemed to postpone the clinical onset of infection, and mortality of birds was lowest in the group of birds vaccinated twice, followed by the group of birds vaccine once, whereas a 100 % mortality of non- autogenously vaccinated group

challenged with the autovaccine strain was observed. Finally, as all birds in all groups had initially been vaccinated with Poulvac®, protection against ascending infections of the two challenge strains in the experiment model could not be documented.

To verify the observed lack of protection against ascending *E. coli* infections provided by Poulvac®, an autogenous vaccine, or a combination hereof, future experimental studies should include dose titration, as protection against lower infection doses may be possible.

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Acknowledgement

The work was supported in by The Danish Poultry levy board project (Highly virulent *Escherichia coli* clones in broiler production) grant nr. 13315.

Figure legends

Figure 1. Scatter plot of cumulative lesion scores for experimental trials with a vaccine strain of *E. coli* (*E. coli* E44) and a vaccine-unrelated strain of *E. coli* (SCI_07) evaluated in an avian model

for infection of oviduct in three different groups of broiler breeders. Maximum lesion score are: Total lesions: 38 (panel A), peritoneum: 15 (B), ovary: 9 (C), and salpinx: 9 (D). Each point represents one chicken within the group. Green circles: Unvaccinated chickens challenged with the vaccine strain *E. coli* E. 44; Red circles: Unvaccinated chickens challenged with the vaccine strain *E. coli* SCI_07; Green triangles: 1x autogenously vaccinated chickens challenged with the vaccine strain *E. coli* E. 44; Red triangles: 1x autogenously vaccinated chickens challenged with SCI_07; Green squares 2x autogenously vaccinated chickens challenged with the vaccine strain *E. coli* E. 44; Green squares 2x autogenously vaccinated chickens challenged with SCI_07. The asterisks indicate statistical significance: * P<0.05; **P<0.01; ***P<0.0001

Figure 2. IgY soluble antibodies against *E. coli* E44 (A), and *E. coli* SCI_07 (B) in broiler breeders challenged with either *E. coli* E44 or SCI_07. Blue bars: Before Challenge; The right adjacent bar: Green: after challenge with *E. coli* E44, Red: after challenge with SCI_07. Au: autogenously vaccinated. The asterisks indicate statistical significance: * P<0.05

Figure 3. IgY soluble antibodies in offspring from broiler breeders vaccinated with an autogenously vaccine including two strains of *E. coli*, *E. coli* E44 and *E. coli* E51, compared to offspring originating from unvaccinated breeders. No significant differences were observed between the level of antibodies originating from unvaccinated versus vaccinated parents for any of the two *E. coli* strains (P=0.3407)

Table 1. Overview of experimental groups. Each group consisted of 2x10 broiler breeders (age 28 weeks)

Group	<i>E. coli</i> autogenous vaccine (week of age)	Challenge strain¹	Average weight at day of challenge	Survived the full experimental period (7 days post challenge)
1A	None	<i>E. coli</i> E44	3699	0/10 ^a
1B	None	SCI_07	3669	10/10
2A	1 time (18)	<i>E. coli</i> E44	3532	8/10
2B	1 time (18)	SCI_07	3529	10/10
3A	2 times (14 and 18)	<i>E. coli</i> E44	3563	9/10
3B	2 times (14 and 18)	SCI_07	3543	10/10

¹ All chickens received a dose of 5×10^5 CFU/mL of the challenge strain. ^a Survival rates were significantly lower than for any of the other groups ($P < 0.0001$)

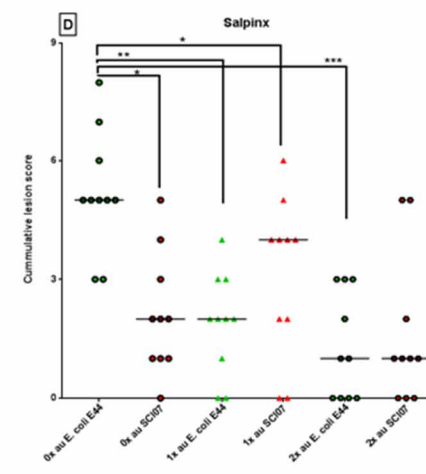
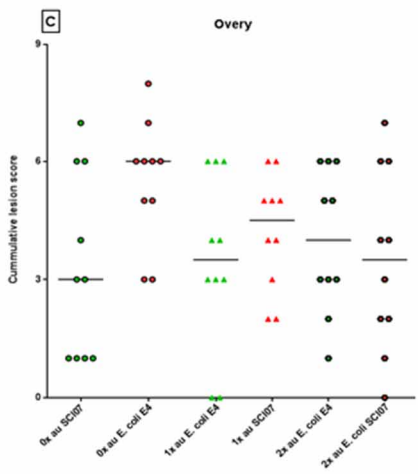
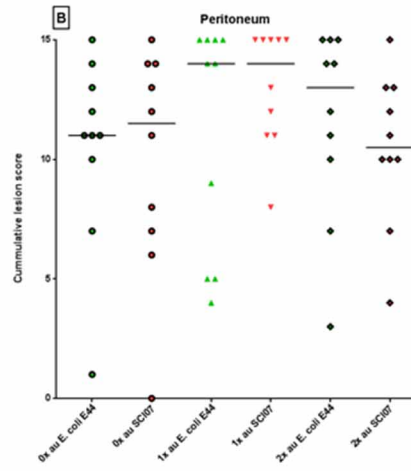
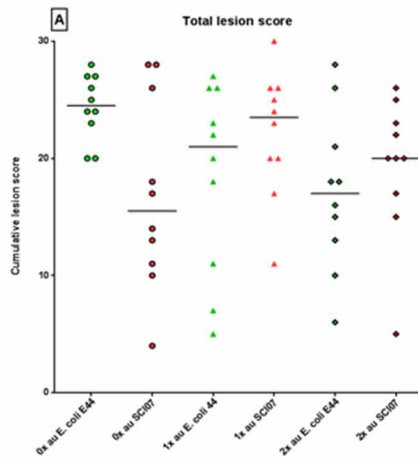
Table 2. Semi-quantitative estimation of bacteria per swab obtained from liver, salpinx and bonemarrow of each chicken. Each group contained 10 chickens

Group	Challenge strain	Growth of <i>Escherichia coli</i> on blood agar plates after 24h incubation at 37° C								
		Liver			Bone marrow			Salpinx		
		Sterile/ Poor growth ¹	Fair growth	Massive growth	Sterile/Poor growth	Fair growth	Massive growth	Sterile/Poor growth	Fair grow th	Massive growth
1A	<i>E.coli</i> E44	0	2	8	2	2	6	0	0	10
1B	SCL_07	10	0	0	10	0	0	6	2	2
2A	<i>E.coli</i> E44	9	0	1	10	0	0	3	2	5
2B	SCL_07	10	0	0	10	0	0	0	3	7
3A	<i>E.coli</i> E44	10	0	0	10	0	0	4	5	2
3B	SCL_07	9	0	0	10	0	0	4	1	5

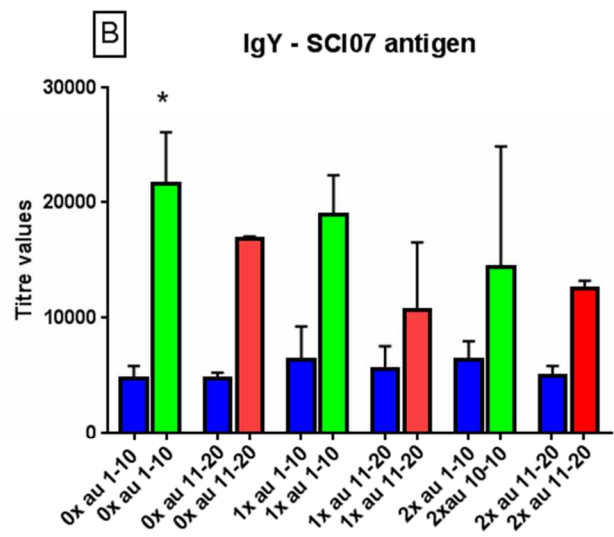
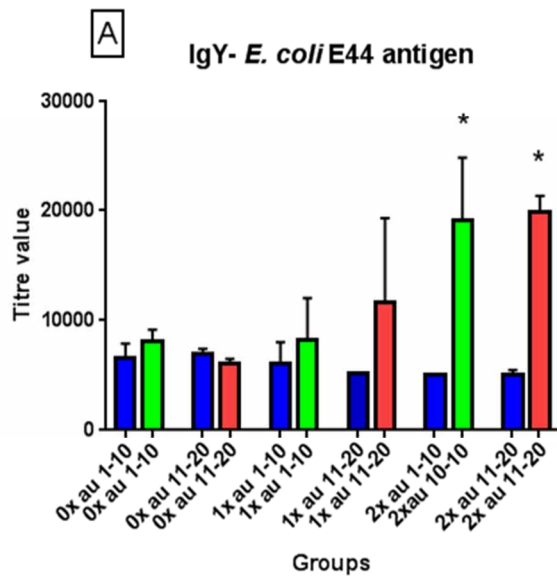
¹ Growth was considered poor if less than 10 colonies of *E. coli* were present on the agar plate after 24h incubation.

Table 3. Overview of mean (median) lesion scores. Scoring was done according to Pors et al. (2014). Maximum score (achieved at highly severe macroscopic pathology) is stated under each category, 0 is given to birds presented with no macroscopically lesions. In addition, the spleen is scored for proliferation and lymphatic reaction (not shown in the table).

Group	Challenge strain	Peritoneum	Ovaries	Salpinx	Total
		[0-15]	[0-9]	[0-9]	[0-38]
1A	<i>E. coli</i> E44	12	5,5	5,2	24
		(11)	(6)	(5)	(25)
1B	SCI_07	8,6	3,3	2,7	16,9
		(12,5)	(3)	(2)	(17,5)
2A	<i>E. coli</i> E44	11,1	3,5	1,8	18,5
		(14)	(3)	(2)	(20)
2B	SCI_07	13	4,2	1,9	22,2
		(14)	(4,5)	(4)	(23,5)
3A	<i>E. coli</i> E44	11,6	4	1,3	19,3
		(13)	(4)	(1)	(20)
3B	SCI_07	10,5	3,4	1,6	17,1
		(10,5)	(3,5)	(1)	(17)

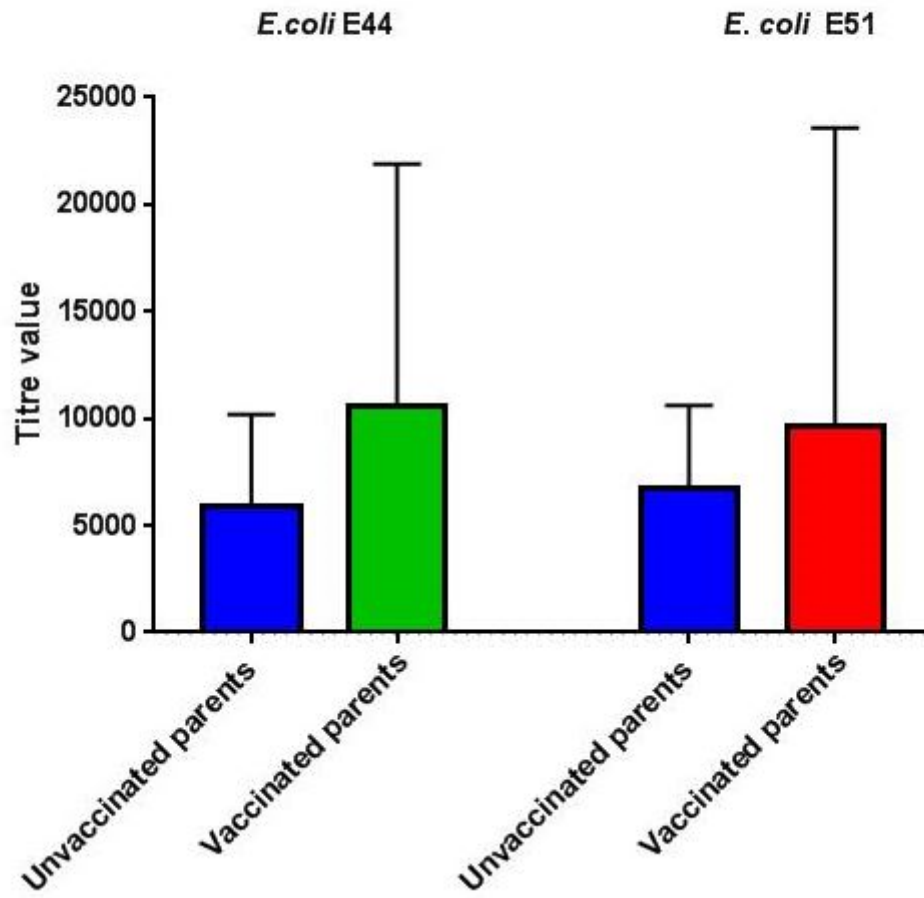


ACCEPTED



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IgY-soube antibodies in offspring



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