UTRECHT UNIVERSITY

Dose-response relationship of ESBL-producing Escherichia coli in broilers

Master Thesis Veterinary Medicine, Dep. Farm Animal Health

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Abstract

The presence of ESBL-producing *Escherichia coli* in the poultry industry might constitute a risk for public health. To reduce these bacteria, interventions should be developed and studied. For this purpose, a valid animal model in which the infection, colonization and excretion patterns of these bacteria in broilers can be studied is needed. The aim of this experiment was to find the lowest dose resulting in gut colonization and to determine the dose-response relationship. In addition, in a field study the ESBL status of a broiler farm was monitored to gain information about the colonization of ESBL-*E. coli* under field circumstances.

The dose-response experiment consisted of three rounds. During each round, broilers were randomly divided over 5 to 6 sterile isolators and were inoculated with different dose levels of ESBL-producing *E. coli*. Cloacal swabs were taken at 1.5 (except for round 1), 3, 6, 9, 12, 15, 20, 24, 28, 32, 48, 52, 56 and 72 hours post inoculation (p.i.) and caecal samples were taken 72 hours p.i.. Samples were tested for presence of ESBL-*E. coli*. For swabs taken at 9, 32, 56 and 72 hours p.i. and caecal content at 72 hours p.i. colony forming unit (CFU)/gram faeces was determined.

The lowest dose resulting in gut colonization was 0.5 mL of a 10^1 CFU/mL suspension. A dose-response relationship was found: a higher dose level resulted in earlier colonization. On average birds were colonized within 10-15 hours after inoculation with the lowest dose (10^1 CFU/mL) and within 1:45-3 hours for the group receiving the highest dose level (10^5 CFU/mL). After colonization, concentration of ESBL-*E. coli* varied between 4.3×10^3 and 12×10^9 CFU/gram faeces. The results indicate that this experimental set-up provides a suitable animal model for further infection studies with ESBL-*E. coli*.

Introduction

The effect of many of the currently used antibiotics depends on beta-lactam, a specific structure in the active molecule. The best known example of a beta-lactam antibiotic is penicillin. However, some bacteria are capable to produce enzymes that inactivate beta-lactam, called beta-lactamases. Such bacteria are resistant against certain antibiotics, making it more difficult to inhibit their growth (Prescott, 2007). Because of this resistance, new antibiotics with an extended spectrum (cephalosporins) were developed, which could inhibit the growth of resistant bacteria. But after a while, some bacteria became resistant to these antibiotics as well. Those bacteria obtained the ability to produce 'extended spectrum beta-lactamases' (ESBLs) (Bradford, 2001). Today, already more than 150 different ESBLs have been described, produced by *Enterobacteriaceae* and *Pseudomonas aeruginosa* (Bradford, 2001) and found in animals, humans and the environment (Blaak *et al.*, 2015).

Several studies describe the current prevalence of ESBLs in broilers. Blaak *et al.* (2015) sampled three broiler farms in The Netherlands and found ESBL-producing *E. coli* on all farms. Huijbers *et al.* (2014) found ESBL-positive samples on all 50 sampled Dutch broiler farms. Dierikx *et al.* (2013) found ESBL-producing *E. coli* in all 26 sampled Dutch broiler farms, of which 22 with a very high prevalence (>80%).

A review of Olsen *et al.* (2014) describes the emergence of ESBL as a serious problem in human medicine. Possibly, contact with chicken or chicken meat is a source of human infection. In a Dutch

study Leverstein-van Hall *et al.* (2011) took 98 chicken meat samples. In 94% of those samples, at least one type of *E. coli* with ESBL phenotype was found. Moreover, some of the ESBLs found on poultry meat were also found in human samples. Therefore they suggest that contact with contaminated poultry meat can possibly be a risk for human infection. In contrast, studies in Germany and Denmark did not find any strong connection between colonization with ESBL-producing *E. coli* in human and contact with poultry meat (Leistner *et al.*, 2013; Carmo *et al.*, 2014).

Blaak *et al.* (2015) proved presence of ESBL-producing *E. coli* in the direct environment of poultry farms. Sixty percent of the selected environmental isolates where equal to the isolates in the flocks. Most isolates where found in soil and surface water. Contact with these contaminated environments may pose a risk to human health as well.

The exact role of farm animals in ESBL transfer to humans remains under discussion until further elucidated. Nevertheless, because ESBLs potentially constitute a risk for public health, it is clear that a reduction of the prevalence of ESBL-producing bacteria in the poultry industry is indispensable. To achieve that goal, interventions in the production chain that may contribute to a reduction of ESBLs should be studied. An animal model may help to elucidate biological mechanisms involved in infection and transmission processes and can be used to test possible interventions to reduce prevalence. Therefore a good animal model has to be developed, in which the infection, colonization and excretion patterns of these bacteria in broilers can be studied. Making such a model starts with mimicking a field infection in a controlled research facility.

An earlier experiment showed that inoculating broilers with 0.5 mL of a suspension with 10^4 colony forming units (CFU) ESBL-producing *E. coli* per mL leads to colonization (Nuotio *et al.*, 2013). However, the model should also reflect the situation on low-prevalence farms, where birds may be exposed to lower doses. Therefore, the aim of this experiment is to find the lowest dose of ESBL-producing *E. coli* resulting in gut colonization after inoculation and to determine the dose-response relationship. Moreover, in this experiment different sampling moments and methods (individual methods like cloacal swabs and caecal contents) were used to investigate what is needed to gather sufficient information regarding ESBL colonization.

In addition to this experiment, in a field study the ESBL status during one entire production round on a broiler farm was examined. This experiment could provide information about (time of) colonization of ESBLs in a flock under field circumstances. Such data can assist with the development of a valid animal model that reflects colonization under field circumstances.

Materials and Methods

Dose-response experiment

Birds

For this experiment, 18-days incubated eggs containing Cobb/Hybro/Ross crossbred broiler chickens were obtained from the specified pathogen free (SPF) parent flock of GD Animal Health Service (Deventer, The Netherlands). The eggs were transported to the animal facilities of the Department of Farm Animal Health of Utrecht University and after disinfection with H_2O_2, incubated in a hatcher. In round 1 of the experiment the eggs originated from a parent flock of 60 weeks of age. For rounds 2 and 3 the eggs originated from a new parent flock of 29 and 32 weeks of age respectively.

Experimental setup

After hatching (day 0) the broilers were tagged and weighed individually and randomly divided over five (rounds 1 and 2) or six (round 3) cleaned and disinfected isolators, resulting in 12-15 chicks per isolator. At day 1, surplus birds were removed and 10 remaining birds were inoculated in the crop with ESBL-producing *E. coli*. Selection of the surplus birds was done based on:

1. Performance. Least vital broilers were removed. If more than 10 broilers remained:
2. Weight at hatch. Birds with weight at hatch under mean weight minus standard deviation were removed. If still more than 10 broilers remained:
3. Random. Broilers were randomly selected as surplus, until 10 birds remained.

Surplus birds were euthanized using cervical dislocation.

Per isolator 10 birds were inoculated orally with 0.5 mL E39.62 with CMY-2 on plasmid IncK or with 0.5 mL phosphate buffered saline (PBS) in the control group. Three similar experimental rounds with different dose levels and amounts of birds were performed. For details of each round, see Table 1.

Table 1. Experimental setup

	Round 1	**Round 2**	**Round 3**
Number of incubated eggs	80	80	120
Age of parent flock*	60 weeks	29 weeks	32 weeks
Dose levels (in CFU/mL)			
Group 1 (control group)	PBS	PBS	PBS
Group 2	10^2	10^1	10^1
Group 3	10^3	10^2	10^1**
Group 4	10^4	10^3	10^2
Group 5	10^5	10^4	10^3
Group 6	-	-	10^5

* Chicks for rounds 2 and 3 originated from the same SPF parent flock.

** From a different serial dilution

Housing

The isolators were 1 m^2 of size and had slatted floors. The floors were covered by thick, waterproof paper with wood shavings (1 kg/m^2) to mimic poultry house environments. Before arrival of the eggs the hatcher and the facility were disinfected with H_2O_2-gas and the isolators with formaldehyde gas. Transport of utensils, feed and animals in and out isolators was done using waterproof containers through a sluice filled with 1% Halamid (tosylchloramide sodium) solution.

Diet

Broilers received *ad libitum* feed (gamma radiated, standard meshed broiler diet without antibiotics or coccidiostats) and water. At the end of the experiment the residual feed per isolator was weighed to estimate the feed consumption.

Sampling

During the experiment, all individual broilers were sampled using cloacal swabs. Samples were taken directly before inoculation and at 1.5 (except for round 1), 3, 6, 9, 12, 15, 20, 24, 28, 32, 48, 52, 56 and 72 hours post inoculation (p.i.) to detect the presence of ESBL-*E. coli* . Concentration of ESBL-*E. coli* and total *E. coli* was determined from the swabs taken at 9, 32, 56 and 72 hours p.i.

At 72 hours p.i., directly after euthanasia, caecal content was collected for analysis of concentration of ESBL-*E. coli* and total *E. coli*.

Performance

Body weight was determined at hatch (day 0) and at the end of the trial (day 4). The feed conversion rate (FCR) was calculated per isolator by dividing the total feed consumption (in grams) by the total growth (in grams). Birds that died before day 4 were excluded and feed consumption was corrected.

End of trial

At day 4, directly after the last swab at 72 hours p.i., the broilers were euthanized using cervical dislocation. The birds were weighed, followed by post-mortem examination. The birds were checked for abnormalities and gender and the caeca were collected.

Sample analysis

- ESBL detection

Directly after taking cloacal samples, samples were processed: swabs were suspended in 3 mL Luria-Bertani (LB) broth. The samples were incubated overnight (for 16-24 hours) at 37^{o}C and thereafter cultured on selective plates (MacConkey +cefotaxime (1 mg/L)). The plates were incubated overnight at 37^{o}C and thereafter assessed visually. Round, dry, purple colonies with a darker spot in the middle were appointed as ESBL-producing *E. coli*.

- ESBL concentration

In part of the samples (swabs of 9, 32, 56 and 72 hours p.i. and in the caecal content) total concentration of *E. coli* and ESBL-producing *E. coli* was determined, using the run and drop method on MacConkey agar and MacConkey + cefotaxime (1 mg/L) plates. After swabbing, each swab was suspended in a tube with 3 mL LB broth. From this suspension 200 µL was transferred to a 1 mL Micronic tube and stored on ice to inhibit bacterial growth during transport. A ten-fold serial dilution was made and six dilutions (10^{0}-10^{-5}) were pipetted on a square MacConkey agar plate with 1 mg/L cefotaxime and on one without cefotaxime. After inoculation each plate was moved to an oblique surface (35-45^{o}) to let the drops run and dry properly. *E. coli* and ESBL-*E. coli* concentrations in the caecal content were determined by making a 10% suspension of 0.1 to 1 gram of the caecal content with PBS. This dilution was used to perform the run and drop method as described above.
The plates were incubated overnight (16-24 hours) at 37^{o}C and thereafter assessed visually. Round, dry, purple colonies with a darker spot in the middle were appointed as *E. coli*.
The swabs were weighed before and after sampling in order to measure the total amount of faeces collected. When swab weight was 0 or lower, the average swab weight of the other swabs from that isolator at that time was used for the calculation. Caecal content was weighed as well.

-Typing at Wageningen Bioveterinary Research, Lelystad, The Netherlands

A selection of in total 200 colonies from different MacConkey plates from different sampling moments was cultured on MacConkey plates with cefotaxime (1 mg/L), to check whether the colonies present on MacConkey plates without cefotaxime contained ESBL genes.
From each isolator seven colonies from the MacConkey plates with cefotaxime were selected and analysed with Random Amplified Polymorphic DNA (RAPD), to compare those colonies with the inoculum.
In rounds 2 and 3, colonies visually assessed as 'not *E. coli*' were identified with MALDI-TOF analysis.

Statistical analysis

Data were entered and processed in Microsoft Excel and transferred to SPSS. The data were checked for normality with Shapiro-Wilk-test and for equality of variances with Levene Statistic. Performance data, i.e. mean body weight at day 0 and day 4, growth and growth per day, were compared between groups and between rounds. One-way ANOVA was done with post-hoc test Tukey and for data with unequal variance post-hoc test Games-Howell was used. FCRs were reported but no statistical analyses were performed because values were rough estimates.

For each broiler the time between inoculation and colonization was registered. Time of colonization was defined as the time of the first ESBL-*E. coli* positive swab in a row of at least two ESBL-*E. coli* positive swabs. Differences between mean times until colonization were analysed with One-Way ANOVA as described above. Kaplan Meier survival curves were made in SPSS based on the individual times between inoculation and colonization. Cox regression with Bonferroni correction was conducted to determine significant differences between groups. Influence of gender and weight on survival curves was tested with Cox regression as well.

The CFU/gram faeces per sample was determined and converted to log(CFU/gram) in Excel and transferred to SPSS. One-Way ANOVA was performed as described above. Samples with a CFU/gram of 0 were excluded for this analysis.

Field trial

Experimental setup

On a conventional commercial broiler farm with historically low antibiotic usage the ESBL status was examined from day 0 to slaughter age (day 40). Two of the four broiler houses, stables 3 and 4, were included in the study, each housing 27,500 Ross 308 broilers. Before arrival, the houses and feed- and water supply were cleaned according to standard hygienic procedures.

Sampling

On day 0 a total of 40 pieces of at least 5x5cm from the paper lining the transport crates, containing faeces of the chicks, were collected per house. Samples were pooled per 20 pieces, resulting in two pools per house.

Houses were sampled using bootsocks that were premoistened in modified Peptone Saline Diluent (Technical Service Consultants, UK). Bootsock samples were collected at days 0, 2, 5, 7, 14, 21 and 40. The first sample (day 0) was taken before the broilers arrived. The floor was visually divided in nine square compartments (3x3, A-I) and in each compartment one bootsock was used, following the walking route in Figure 1. Disposable gloves and shoe covers were used and changed after sampling of each compartment.

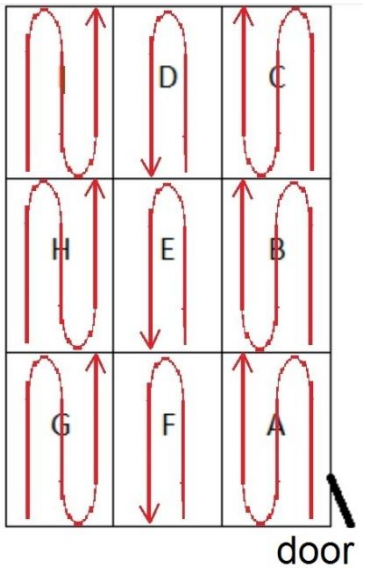

Figure 1. Walking route bootsock sampling

Sample analysis

All individual bootsock samples and pooled paper samples were mixed with 250 mL LB broth with cefotaxime (1 mg/L) and incubated at 37°C for 16-24 hours. After incubation, 10 µL of the broth was plated on MacConkey + cefotaxime (1 mg/L) plates and incubated at 37°C for 16-24 hours. Plates were visually assessed and colonies were identified as *E. coli* by typical appearance.
MALDI-TOF analysis was performed at days 14 and 40 to confirm the visual judgement. Both typical and non-typical colonies were included for identification. At day 14, a MALDI-TOF analysis on 26 colonies from plates of different days was performed. The second MALDI-TOF was performed on the samples of day 40. This time, from the agar plates of all individual bootsocks the appearance was assessed for all colonies. Per plate, from each type of colonies, one colony was selected and inoculated on a new blood agar plate and incubated at 37°C for 16-24 hours. Colonies from these blood agar plates were used for MALDI-TOF analysis.

Results

Dose-response experiment

Per round the average weight at hatch was respectively 45.8 g, 37.3 g and 39.6 g and at day 4 respectively 94.9g, 79.5g and 84.2g. In all three rounds the gender recorded during post-mortem examination indicated more male than female broilers. Mortality was low. During round 2, mean weight at hatch of group 4 significantly differed from the mean of group 5. No other differences between mean weight at hatch, weight at day 4, growth and growth per day were found between groups in all rounds. Mean weight at hatch, weight at day 4 and growth per day significantly differed between the three rounds: those were significantly higher in round 1 than in rounds 2 and 3 and in round 3 significantly higher than in round 2. Mean growth in rounds 1 and 3 were significantly higher than in round 2. For each round details are described in Table 2.

Table 2. Summary statistics

Round 1	**Total**	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 5**
Dose level (CFU/mL)		PBS	10^2	10^3	10^4	10^5
Average weight at hatch (g)	45.8	46.8*	47.4*	46.3*	46.8*	47.5*
Average weight at day 4 (g)**	94.9	94.0	96.2	96.4	90.2	96.8
Average growth (g)**	47.9	47.4	48.8	50.1	43.1	49.3
Average growth/day (g)**	12.0	11.8	12.2	12.5	10.8	12.3
#chicks died	3	2	0	0	1	0
#males/females	36/12	6/2***	10/0	8/2	6/4	6/4
FCR	2.15	1.94	2.28	2.33	2.01	2.14

Round 2	**Total**	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 5**
Dose level (CFU/mL)		PBS	10^1	10^2	10^3	10^4
Average weight at hatch (g)	37.3	38.4*	37.3*	38.3*	39.3*^{a}	36.9*^{b}
Average weight at day 4 (g)**	79.5	80.2	78.3	79.2	78.4	80.9
Average growth (g)**	41.5	41.8	41.3	40.9	39.0	44.0
Average growth/day (g)**	10.4	10.4	10.3	10.2	9.8	11.0
#chicks died	7	1	2	0	3	1
#males/females	37/13	8/2	7/3	8/2	7/3	7/3
FCR	1.68	1.90	1.88	1.55	1.38	1.67

Round 3	**Total**	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**
Dose level (CFU/mL)		PBS	10^1	10^1	10^2	10^3	10^5
Average weight at hatch (g)	39.6	39.7*	40.0*	39.8*	40.4*	39.5*	39.4*
Average weight at day 4 (g)**	84.2	81.5	84.8	84.0	88.9	85.1	80.9
Average growth (g)**	44.4	41.8	44.8	44.2	48.5	45.7	41.2
Average growth/day (g)**	11.1	10.5	11.2	11.1	12.1	11.4	10.3
#chicks died	2	0	0	0	0	1	1
#males/females	37/22	7/3	7/2***	7/3	7/3	5/5	4/6
FCR	1.90	2.21	1.84	2.02	2.00	1.60	1.71

	Round 1	**Round 2**	**Round 3**
Number of eggs hatched	72	74	110
Average weight at hatch (g)	45.8^a	37.3^b	39.6^c
Average weight at day 4 (g)**	94.9^a	79.5^b	84.2^c
Average growth (g)**	47.9^a	41.5^b	44.4^a
Average growth/day (g)**	12.0^a	10.4^b	11.1^c

*Broilers euthanized before inoculation are not included

** Broilers that died before day 4 are not included

*** The gender of some broilers was unknown

Data in the same row with different symbols (a,b,c) significantly differ ($p<0.05$)

ESBL detection

None of the samples taken before inoculation contained ESBL-producing *E. coli* in any round. For each chick, time between inoculation and colonization was determined. On average birds were colonized within 10-15 hours after inoculation with the lowest dose (10^1 CFU/mL) and within 1:45-3 hours in the group receiving the highest dose level (10^5CFU/mL).

In round 1, time until colonization for the group with dose level 10^2 CFU/mL was significantly longer than for the groups with higher dose levels. Also in round 2 time until colonization was significantly longer in the group with the lowest dose compared to other groups. In round 3, in the three groups with dose levels 10^1 and 10^2 CFU/mL time until colonization was significantly longer compared to the groups with dose levels 10^3 and 10^5 CFU/mL.

Figure 2 shows the link between the dose of the inoculum and the mean time from inoculation until colonization. Means and significance are shown in Table 3.

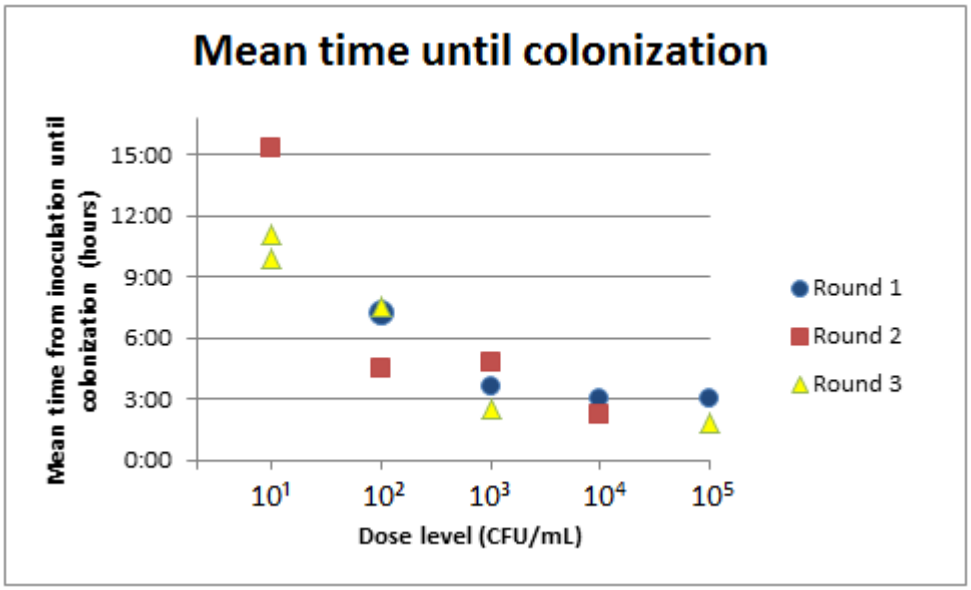

Figure 2. Mean time until colonization for the different dose levels

Table 3. Mean time until colonization

	10^1 CFU/mL	10^2 CFU/mL	10^3 CFU/mL	10^4 CFU/mL	10^5 CFU/mL
Round 1	x	7:12h[a]	3:36h[b]	3:00h[b]	3:00h[b]
Round 2	15:18h[a]	4:30h[b]	4:48h[b]	2:15h[b]	x
Round 3	10:30h[a]*	7:30h[a]	2:33h[b]	x	1:48h[b]

* Groups 2 and 3 combined. Group 2 9:54h, Group 3 11:06h

Data in the same row with different symbols (a,b) significantly differ ($p<0.05$)

Time until colonization is shown in Kaplan Meier graphs (Figures 3, 4 and 5). In those figures, the percentage on the y-axis represents the percentage of birds that is not yet colonized by ESBL-*E. coli*. In rounds 1 and 3, after 15 hours 100% of the birds was colonized. In round 2 this took 24 hours. During round 1 no significant differences between the survival curves of the groups are seen. In round 2, the curve of the group with dose level 10^1 CFU/mL was significantly different from the other three curves. In round 3, the curves of dose level 10^1 and 10^2 CFU/mL differed from the curves of 10^3 and 10^5 CFU/mL. Gender or weights did not have significant influence on time until colonization.

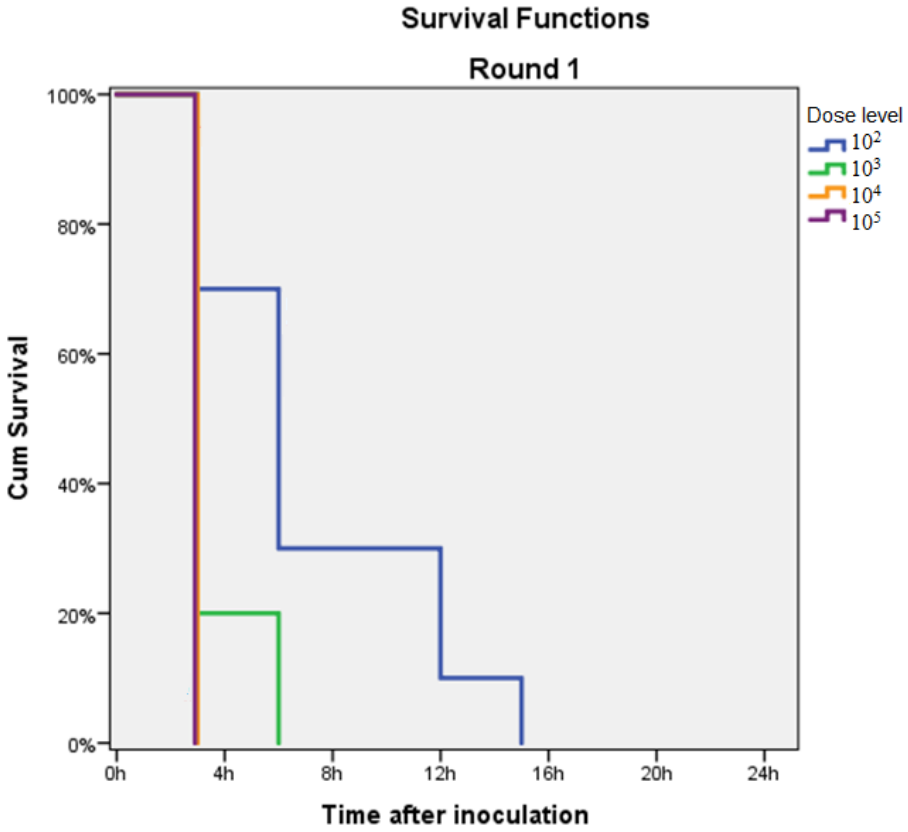

Figure 3. Survival curves of different dose levels in round 1. Curves are not significantly different.

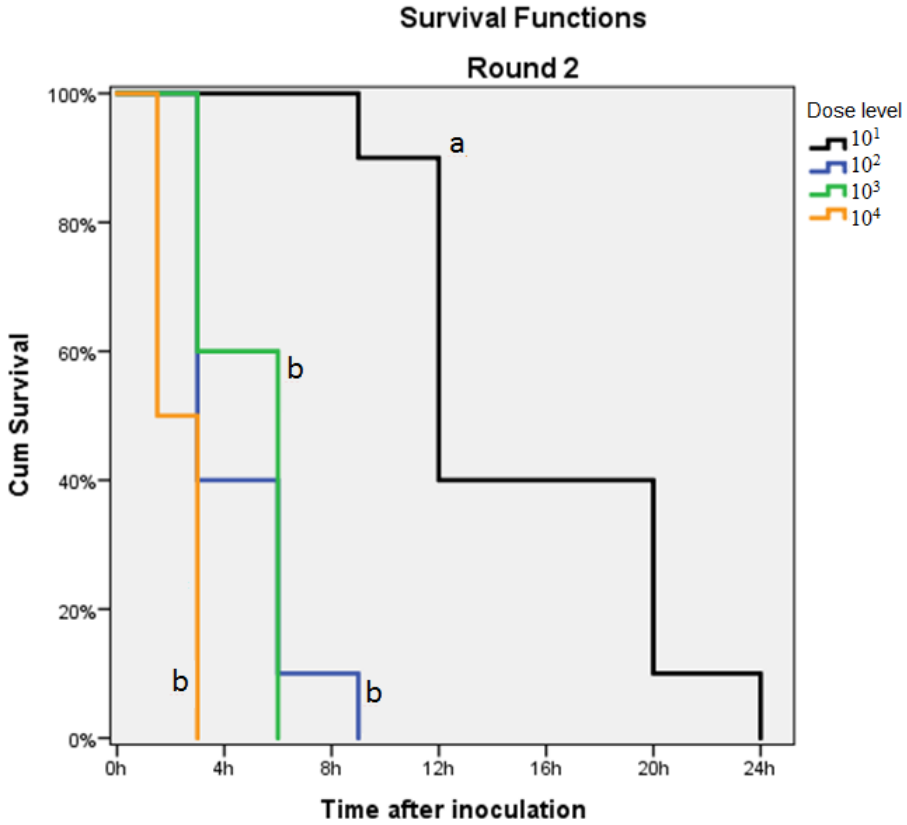

Figure 4. Survival curves of different dose levels in round 2. Curves with different symbols (a,b) significantly differ ($p<0.01$).

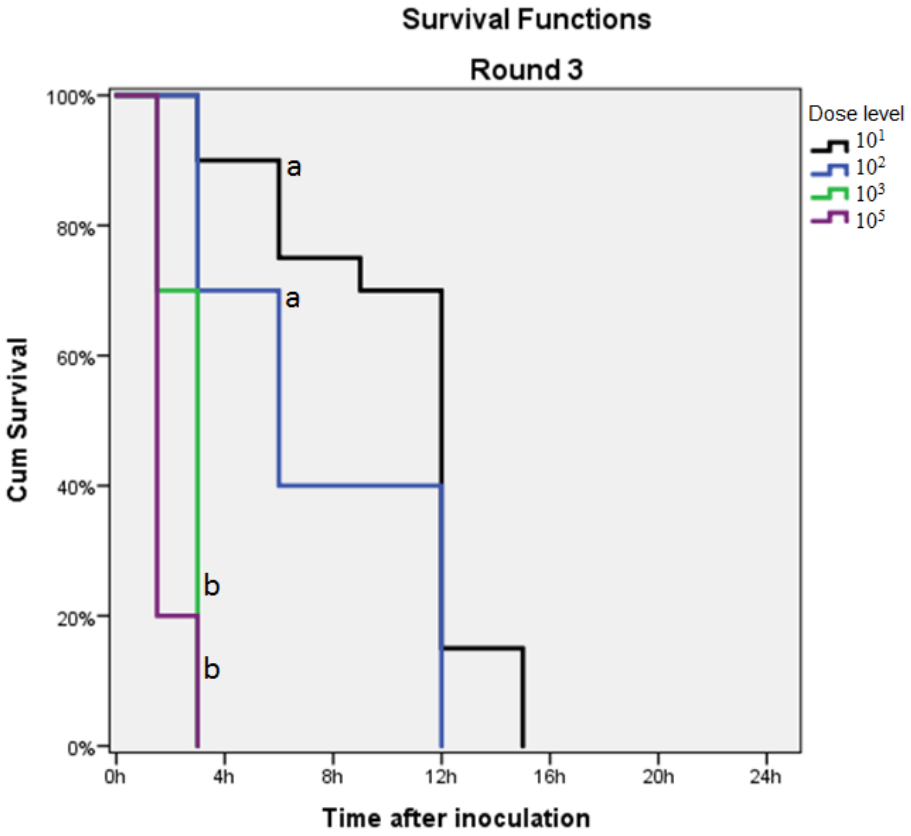

Figure 5. Survival curves of different dose levels in round 3. Curves with different symbols (a,b) significantly differ ($p<0.01$). Results from groups 2 and 3 are combined.

ESBL quantification

At 9 and 32 hours p.i. some samples were negative. In all rounds, at 56 and 72 hours p.i. all inoculated birds excreted ESBL-*E. coli*. In positive samples, concentration of ESBL-*E. coli* varied between 4.3 x 10^3 and 12 x 10^9 CFU/gram faeces. The course of excretion of ESBL-*E. coli* in colonized birds is visualized in boxplots (Figures 6, 7 and 8).

Mean log(CFU/gram) and significance ($p<0.05$) are shown in Table 4. No significant differences between means were found in rounds 1 and 2. During round 3, at 9 hours p.i. the group with dose level 10^2 CFU/mL had a significantly lower excretion than the group with dose level 10^5 CFU/mL. In contrast, at 72 hours after inoculation, birds inoculated with the lowest dose of 10^1 CFU/mL had a significantly higher excretion than birds which received the dose level 10^2 CFU/mL.

Data of each group on each sampling moment were checked for normality. Not normally distributed data are indicated with an asterisk in Table 4.

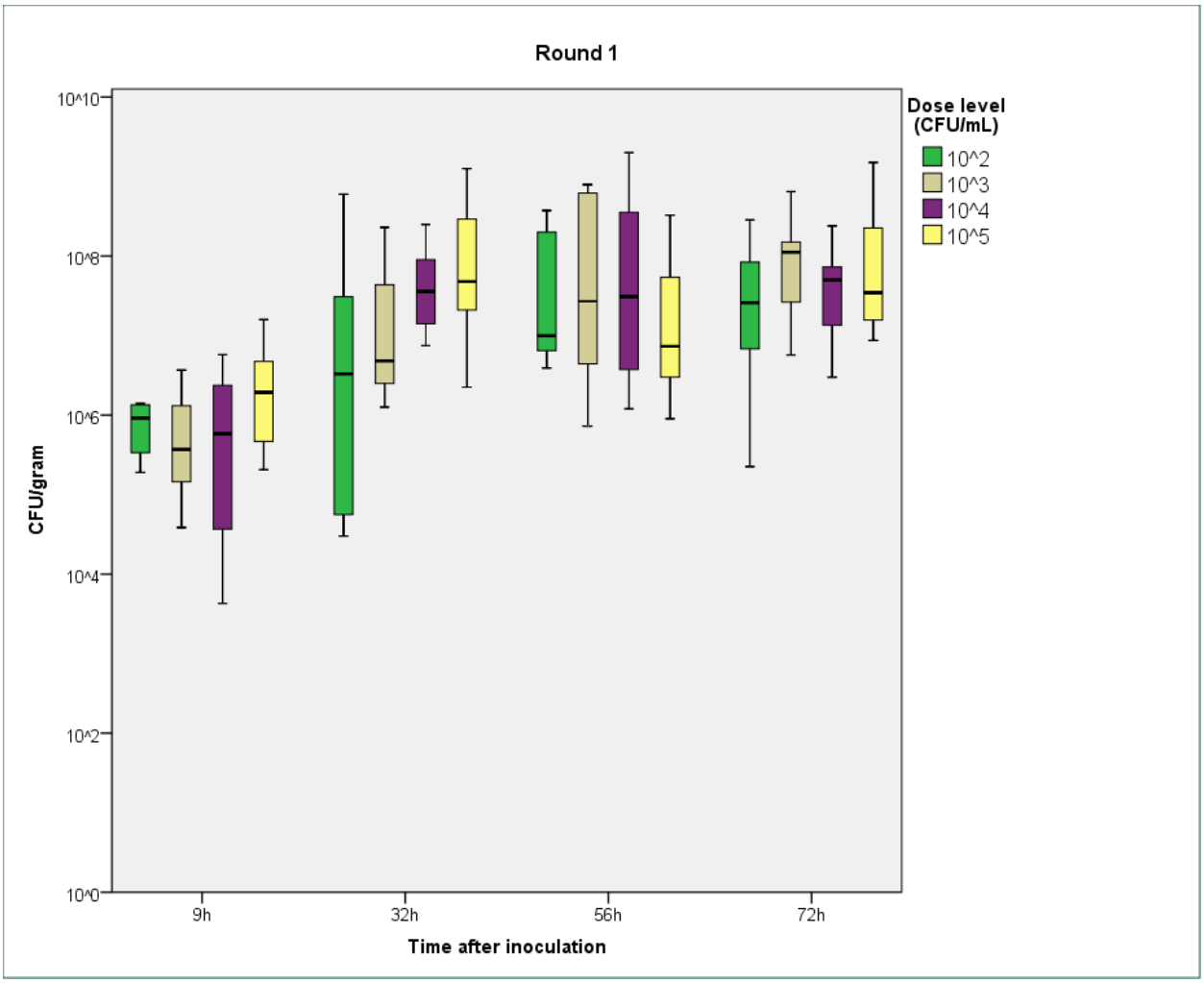

Figure 6. Excretion round 1

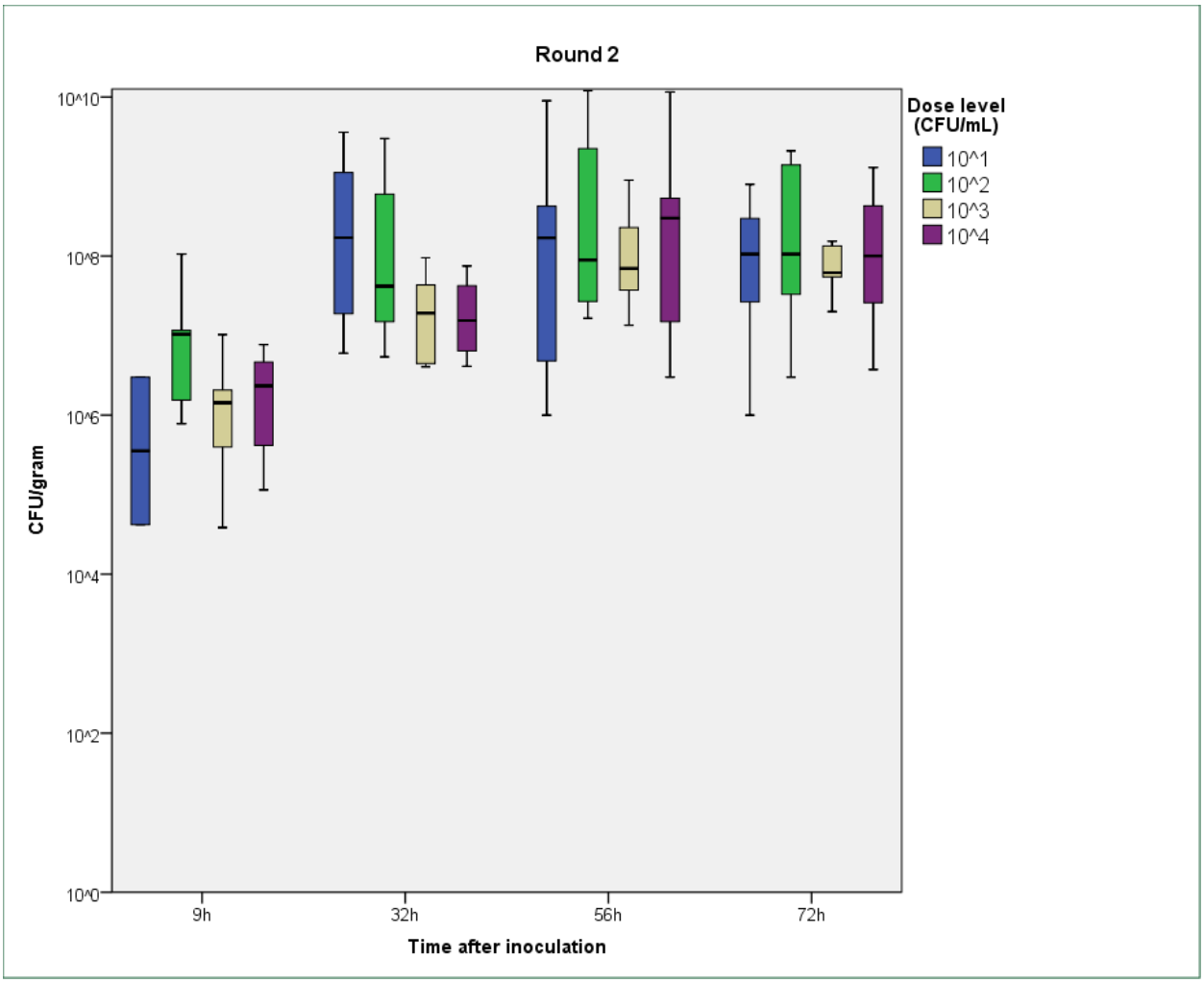

Figure 7. Excretion round 2.

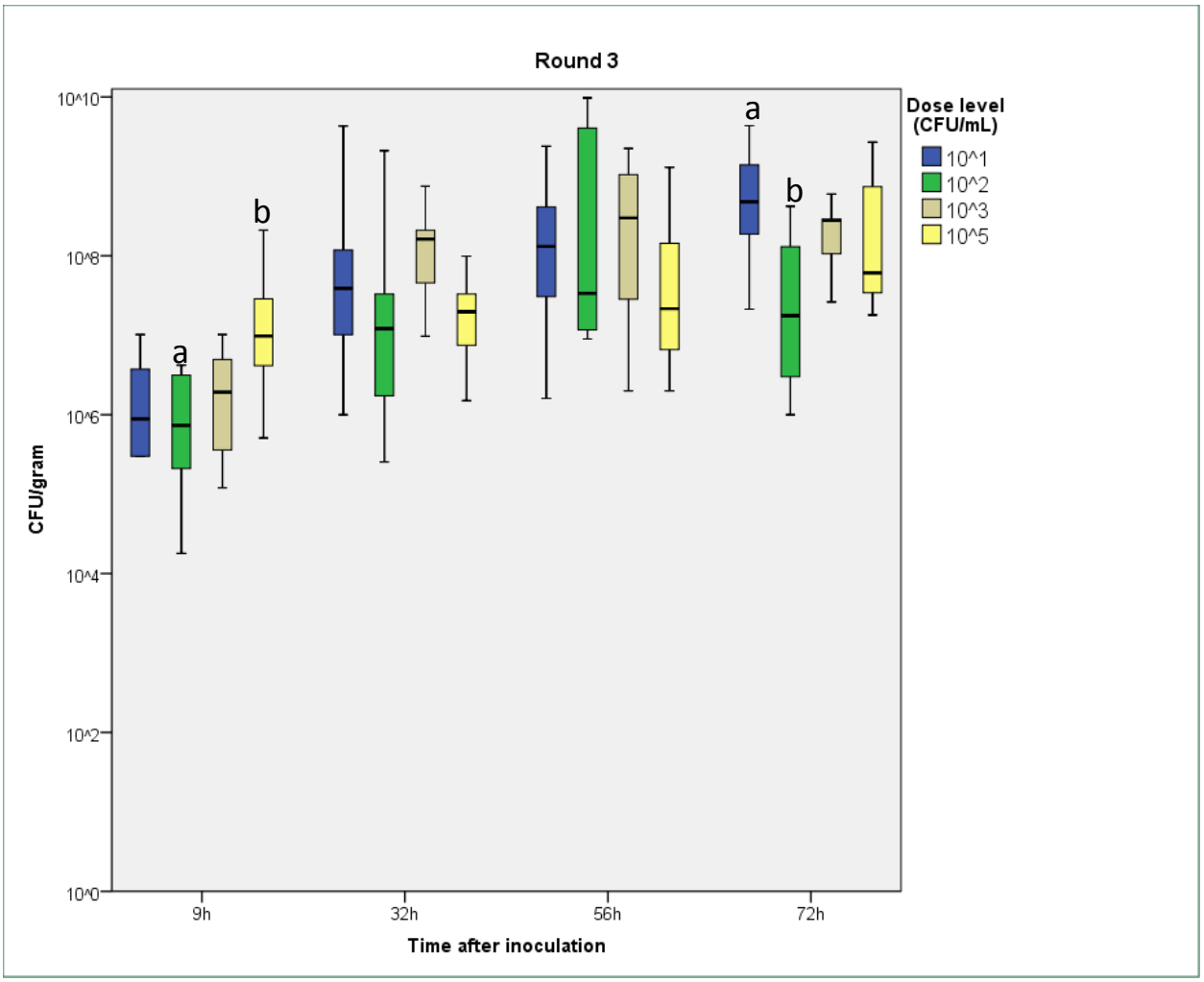

Figure 8. Excretion round 3. Boxplots at the same time with different symbols significantly differ (p<0.05). Results from groups 2 and 3 are combined.

Table 4. Mean log(CFU/gram) per inoculum, time and round

Round 1	**CFU 10^2**	**CFU 10^3**	**CFU 10^4**	**CFU 10^5**
9h	5.872	5.528	5.475	6.276
32h	6.321	7.027	7.554	7.739
56h	7.360*	7.494	7.544	7.075
72h	7.232	7.893	7.488	7.782
Round 2	**CFU 10^1**	**CFU 10^2**	**CFU 10^3**	**CFU 10^4**
9h	5.550	6.778	5.940	6.024*
32h	8.174	7.910	6.872	7.217
56h	7.879	8.264	7.970	8.251
72h	7.843	8.134	7.859	7.893
Round 3	**CFU 10^{1}****	**CFU 10^2**	**CFU 10^3**	**CFU 10^5**
9h	6.072	5.738^a	6.115	7.020^b
32h	7.705	6.976	8.091	7.222
56h	8.023	8.024*	8.209	7.553
72h	8.599^a	7.342^b	8.217	8.133

* Data not normally distributed

** Results from groups 2 and 3 are combined

Data in the same row with different symbols (a,b) significantly differ (p<0.05)

Field trial

At day 0 none of the plates from the bootsock samples contained typical *E. coli* colonies. But in stable 4, both the pooled paper samples did contain cefotaxime resistant colonies with typical *E. coli* appearance. During the first two weeks, colonies from different compartments in both stables were visually assessed as cefotaxime resistant *E. coli*. At days 14, 21 and 40 all samples were assessed as cefotaxime resistant *E. coli* positive. However, MALDI-TOF analysis showed that only 1/9 analyzed samples of the first week was *E. coli* and none of the paper samples contained *E. coli*. The samples consisted of different other cefotaxime resistant gram-negative bacteria. The MALDI-TOF did confirm presence of *E. coli* in all analyzed bootsock samples from day 40. The results are shown in Table 5.

Table 5. Results field trial

Section	Day 0		Day 2		Day 5		Day 7		Day14	Day21	Day 40	
	Vis.	MALDI-TOF	Vis.	MALDI-TOF	Vis.	MALDI-TOF	Vis.	MALDI-TOF	Vis.	Vis.	Vis.	MALDI-TOF
3A	-	*Enterobacter cloacae*	?		?		?		+	+	+	*E. coli, P. aeruginosa*
3B	-	*A. radioresistens*	?		?		+		+	+	+	*E. coli, A. gerneri*
3C	-		?		?		?		+	+	+	*E. coli, A. baumannii, P. aeruginosa*
3D	-		?		+		+		+	+	+	*E. coli, P. aeruginosa*
3E	-		-		+		+		+	+	+	*E. coli, A. gerneri*
3F	-		+	*C. freundii*	+		+		+	+	+	*E. coli, P. aeruginosa*
3G	-		-		+		?		+	+	+	*E. coli, P. aeruginosa*
3H	-		+		+	*A. baumannii*	?		+	+	+	*E. coli, P. aeruginosa*
3I	-		?		?		+	*A. baumannii, P. aeruginosa*	+	+	+	*E. coli, P. aeruginosa*
4A	-		-	*A. baumannii*	+		+	*A. baumannii, E. coli*	+	+	+	*E. coli, P. aeruginosa*
4B	-		-		+		+		+	+	+	*E. coli*
4C	-		-		+	*A. baumannii, Enterobacter cloacae*	+		+	+	+	*E. coli, P. aeruginosa*
4D	-		?		+		+		+	+	+	*E. coli*
4E	-		-		+		+		+	+	+	*E. coli*
4F	-	*A. baumannii, P. putida or P. fulva*	-		+		+		+	+	+	*E. coli, A. baumannii*
4G	-		-		+		+		+	+	+	*E. coli, P. aeruginosa*
4H	-		-		+		+		+	+	+	
4I	-		-		+		+		+	+	+	

Paper	Vis.	MALDI-TOF
3.1	-	*A. baumannii, P. mosselii*
3.2	-	
4.1	+	
4.2	+	*Klebsiella pneumoniae*

Vis.: Visual assessment

+: Colonies with typical *E. coli* appearance found

- : No colonies with typical *E. coli* appearance found

?: No judgement possible with visual assessment

A.: *Acinetobacter,* C.: *Citrobacter*, E.: *Escherichia*, P.: *Pseudomonas*

Conclusion and discussion

Dose-response experiment

The aim of the experiment was to find the lowest dose of ESBL-producing *E. coli* resulting in colonization. This study demonstrated that administration with 0.5 mL of suspension containing 10^1 CFU/mL ESBL-*E. coli* can already result in colonization within a few hours.

The results show that there is a clear dose-response relationship. In all rounds, increasing dose levels showed a significant decrease in time until colonization. In contrast, the relationship between dose and excretion was less clear. In round 3, at 9 hours p.i. a lower dose level resulted in lower excretion. Oppositely in round 3 at 72 hours p.i. the lowest inoculum resulted in a higher excretion than the tenfold higher dose. Those results may indicate a slight trend that a higher dose level causes more excretion in the first hours of colonization. However, at 72 hours p.i. this was definitely not the case anymore, shown by the opposite significant results of round 3. This significant difference does not indicate an opposite relation after 72 hours but was presumably an incidental finding.

In this study it is assumed that actual colonization due to inoculation has occurred, when this has resulted in presence and multiplication of microorganisms. Finding the *E. coli* species of the inoculum in the cloacal swab, does not necessarily mean that actual colonization took place. It is possible that the bacteria passed the digestive system without multiplication. Some birds in the experiment showed one positive result, followed by one or more negative swabs. Therefore, time of colonization in this experiment was defined as the first positive swab in a row of at least two positive swabs. This was based on the assumption that when two consecutive swabs are positive, actual colonization rather than mere passage of the inoculum has occurred. Whether this is a valid assumption cannot be determined with the results of this experiment. Therefore it remains uncertain whether the first positive swab for some birds was the result of passage or colonization and whether the second positive swab indeed indicates colonization.

There are some limitations about the study left to discuss. The run and drop method was used for quantitative measurement of excretion. After sampling the swabs were diluted in medium that contains nutrients for bacterial growth. Those samples were directly placed on ice, but some bacterial multiplication cannot completely be ruled out. After incubation, each row on the plate should contain a tenfold of colonies of the previous row, but that was not always the case. It is uncertain which of the rows then represents the concentration of bacteria in that particular sample. In addition, some errors can be made when counting the number of colonies, for example when they overlap. Although this method provides an indication of the number of excreted bacteria, it is not very exact so conclusions about relatively small differences between groups should be taken with caution.

The nutrients in the LB medium support growth of *E. coli*, but other bacteria can grow unrestrained in this medium as well. When small amounts of *E. coli* are accompanied by larger amounts of other bacteria, this might lead to overgrowth and false-negative results. To reduce overgrowth by bacteria sensitive to cefotaxime, enrichment of the LB medium with cefotaxime should be considered for future experiments.

Another potential source of uncertainty is the amount of bacteria in the inocula. The inocula were gently shaken between each inoculated bird and each inoculum was tested on blood agar plates to verify the number of CFU/mL. Nevertheless, the numbers 10^1, 10^2, 10^3, 10^4 and 10^5 CFU/mL are probably not the exact amounts that will be inoculated in each of the chicks in the isolator. Inevitably there will be some variation in the amounts between the chicks in one isolator.

Also, some differences between the rounds could be observed. These can possibly be explained by the difference in parent flock. The birds in round 1 were from 60 week old broiler breeders. The birds in round 2 and 3 were from another breeder flock, which was younger and probably with slightly different genetics. A part of the experiment was performed in summer, so difference in temperatures can also be an explaining factor for differences between rounds.

In this experiment scientists and animal caretakers knew what dose level was given to each group. Although it is unlikely that this lack of blinding to the experimental group has affected the results, it cannot be excluded that groups were treated slightly different.

It should be noted that this experiment was conducted with SPF birds. These SPF broilers may differ from commercial broiler chickens with regard to intestinal bacteria present at hatch and intestinal physiology. Therefore, the results of these experiments cannot be simply extrapolated to field circumstances. Also, the broilers were housed in sterile isolators. To create an animal model which reflects field circumstances, more research needs to be done, using commercial broilers housed in an environment which better reflects the situation in a broiler house. Presence of other bacteria in the environment can lead to gut colonization with different species, which probably leads to a different pattern of infection, colonization and excretion and a different dose-response reaction of *E. coli*.

With the current results it seems that oral inoculation in the crop with ESBL-*E. coli* is an appropriate way to infect broilers for experimental purposes. However, other natural inoculation routes (via feed or environment) should be studied. This will make repeatable and exact inoculation with a certain dose level even more difficult though.
Ideally, the animal model to study interventions contains commercial broilers in field-like environments. This experiment showed that even the smallest amount of bacteria causes high levels of excretion. This suggests that a successful intervention should prevent colonization after inoculation with a very low dose or completely prevent the chicken against any exposure to ESBL-producing *E. coli*.

Field trial

The field trial was a very limited study, performed on only one farm during one production round. In that round, no cefotaxime resistant *E. coli* was found on days 0, 2 or 5. Different samples were visual assessed as *E. coli*, but the MALDI-TOF confirmed only one visual assessment as *E. coli*, therefore the visual assessment is considered to be unreliable for this type of samples. With MALDI-TOF analysis, presence of cefotaxime resistant *E. coli* was shown on day 7 in one sample and on day 40 in all analyzed samples.

In the first day's samples, no *E. coli* was found, in the paper nor in the litter, so the source of the cefotaxime resistant *E. coli* is unknown. Maybe a certain amount of broilers carried the bacteria in their gut without measurable excretion or it was present at the farm and the broilers became colonized later during the round. Another possibility is that the *E. coli* was excreted, but other

cefotaxime resistant bacteria were excreted as well and overgrew the *E. coli* in the LB broth or on the agar.

Before the start of the experiment, we did not expect to find all those different cefotaxime resistant gram-negative bacteria. When this experiment would be repeated, MALDI-TOF analysis should be considered as test method for all samples.

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