Pilot: Biotyping *E. cecorum* strains using a Chicken Embryo Lethality Assay

Master thesis, Veterinary Medicine, Utrecht University
L.F.P. Snoeijen, 5614473, February, 2021
Project tutor: T.T.M Manders

Abstract

Since 2002 there is an increase in incidence of *Enterococcus cecorum* lesions in chicken. Typical *E. cecorum* lesions are pericarditis, arthritis, osteomyelitis of the femur and 6th thoracic vertebrae and subsequently lead to clinical signs such as weakness, lameness, paralyses and a typical posture due to compression of the spinal cord. To investigate different strains of *E. cecorum* this pilot was performed to determine if an embryo lethality assay (ELA) is a suitable assay to determine if an *E. cecorum* is non-pathogenic or pathogenic. Various routes of inoculations were investigated to determine the success rates of inoculation and the route which can discriminate between the pathogenic and non-pathogenic *E. cecorum* strains. The *E. cecorum* ELA has been performed with allantoic cavity (AC), chorionallantoic membrane (CAM) and intravenous inoculation. The success rates of the amniotic, embryonic and yolk sac inoculation never reached a full score and therefore were not included. The ELA was performed on incubation day 10 and day 14 with 6 different *E. cecorum* strains and a control group. The AC inoculation resulted in a significant difference in survival between one pathogenic strain and the other inoculated strains, with the exception of the negative control strain. The CAM and IV inoculation resulted in a significant difference in survival between the inoculated pathogenic and non-pathogenic strain and the negative control ECE 3 strain. Based upon this pilot study, the CAM inoculation is currently the best inoculation route to discriminate between a pathogenic and a non-pathogenic *E. cecorum* strain in an embryo lethality assay. In addition, the AC inoculation route also provides evidence that it can be a reproducible and reliable method to discriminate *E. cecorum* strains.

Keywords

Enterococcus cecorum; embryo lethality assay; chorion-allantoic-membrane; inoculation; pathogenicity.

Introduction

Since 2002 *Enterococcus cecorum* is more often isolated from lesions in chicken[1]. As one of the *Enterococcus* spp. that is associated with disease, *E. cecorum* became clinically important throughout the last 2 decades[1]. With case reports from several European countries, Canada, The United states and South Africa, *E. cecorum* is a worldwide challenge and threat to the health of chicken flocks[2]. The first literature dates back to 1983, when Devriese *et al.* for the first time isolated a *E. cecorum* strain[3]. From 1983 until present, the literature mainly consists of case reports with *E. cecorum* infections in chickens, ducks and pigeons[1]. The clinical signs associated with an *E. cecorum* infection in chickens are weakness, lameness, paralyse and a typical posture due to compression of the spinal cord. Typical lesions of *E. cecorum* can be found post mortem, such as pericarditis, (septic) arthritis and osteomyelitis of the femur and 6th thoracic vertebrae[1,4–6]. Ultimately, *E. cecorum* infections result into increased mortality rates, up to 15% within broiler flocks, and high rates of condemnation at slaughterhouse due to scratching and dehydration[2,7,8].

In several studies the phenotypes and genotypes between non-pathogenic and pathogenic *E. cecorum* strains are compared and analysed. Borst *et al.* (2015) used comparative genomic and molecular phylogenetic analysis and found that the pathogenic strains had smaller genomes, higher guanine-cytosine rates in comparison with non-pathogenic isolates. In addition, several proteins (n=414) were highly conserved in pathogenic strains and not present in non-pathogenic strains[9]. Dolka *et al.* (2017) used gene sequencing and pulsed field gel electrophoresis to compare 148 isolates and also concluded a genetic heterogenicity among pathogenic strains[10]. In the past, embryo lethality assays (ELA) have been used to determine the virulence of bacterial strains[11–13].

The literature regarding ELA and performed ELAs describe several different routes of inoculation. The two best defined and most common performed inoculation routes are the allantoic cavity (AC) inoculation and chorion-allantoic-membrane (CAM) inoculation [5,10,12–15]. However, amniotic cavity/embryonic (AE) inoculation, yolk sac (YS) inoculation and intravenous (IV) inoculation are also described in various manuscripts[15–18]. Borst *et al.* (2014) compared 8 pathogenic *E. cecorum* strains with 9 non-pathogenic *E. cecorum* strains using an ELA inoculating into only the allantoic cavity. Significant differences in embryonic survival rates between pathogenic and non-pathogenic *E. cecorum* strains were obtained. However, it was not possible to classify the *E. cecorum* strains as pathogenic

or non-pathogenic solely based on this ELA. In this study only egg of incubation day 12 were used. The results might depend on the embryonic age, as this is highly associated with the maturation of the embryonic immune system[19]. The goal of this pilot was to detect difference in virulence of potential non-pathogenic and potential pathogenic *E. cecorum* strains. Various inoculation routes, *i.e.* amniotic cavity or embryo, allantoic cavity, CAM and yolk sac, on embryonic day 10 and 14 were used as various survival rates between the used strains might be influenced by the route of inoculation. This pilot consisted of two parts: 1. Success rates of the inoculations of the various compartments were assessed. 2. The embryo lethality of chicken embryos after inoculation of various *E. cecorum* strains were assessed after 10 and 14 days of incubation. For this pilot we hypothesized that the embryos infected with pathogenic strains of *E. cecorum* would show decreased survival rates in comparison with the embryos infected with non-pathogenic *E. cecorum* strains.

Methods and materials

Part I – success rates of inoculation

During the first part, the AE, YS and IV inoculations were performed by inoculating a blue dye (methylene blue) to determine the success-rates of inoculation. Directly after the inoculation the deposition site of the methylene blue was assessed by visual inspection. Twenty embryonated eggs incubated for 10, 12, 14, 16, 17 and 18 days were inoculated by using the AE inoculation method. For the YS and IV inoculation, the eggs were incubated for 10 and 14 days. For the IV inoculation 10 eggs per group and for the YS inoculation 2 groups of 20 eggs were used. In total 180 eggs were inoculated during the first part.

Part II – Embryo lethality assay

An ELA was performed during the second experiment of this pilot. For the second part 460 embryonated eggs were available for inoculation. All 7 strains (see below) were inoculated into the AC, while 3 strains, *i.e.* ECE 1, ECE 2 and ECE 3, were inoculated onto the CAM. All inoculations were performed on 10 and 14 day incubated eggs. Foregoing results in twenty groups, per group 20 embryonated eggs each were and therefore 400 embryonated eggs were used. The remaining embryonated eggs were used as follows: 4 groups of 15 embryonated eggs each were inoculated IV. Strains ECE 1, ECE 2 and ECE 3 were inoculated in 10 day incubated eggs and strain ECE 3 was inoculated in 14 day incubated eggs. Unsuccessful inoculated eggs were discarded, the number of successful inoculated eggs are shown in table 4. The ELA was performed blindly to eliminate experimental bias.

Eggs. The chicken eggs used in both experiments were collected from commercial Ross 308 broiler breeders between 30-40 weeks of age. The broiler breeders had no history with any *E. cecorum*-associated disease. The collected eggs were stored at 18°C for a maximum of 10 days prior to incubation. The incubators were set at 37.5°C and a relative humidity of 55% for both parts of this pilot. Prior to the inoculation, eggs were candled and all non-viable and infertile eggs were removed. Just before inoculation, the eggs were randomly assigned to a group and marked. To prevent cooling down of the embryonated eggs, the time outside the incubator was kept as short as possible.

Table 1. detailed overview of the E. cecorum strains used in the pilot. Pathogenicity is based on the location of isolation.

ECE strain	Mannitol metabolism	Strain classification[1]	Origin of the sample	Macroscopic lesion	Type of animal	Age of birds (weeks)
ECE 1	negative	Non-pathogenic	Cloaca		Broiler breeder	60
ECE 2	negative	Pathogenic	6th thoracic Vertebra	osteomyelitis	Broiler	7
ECE 3						
ECE 4	positive	Non-pathogenic	Cloaca		Broiler breeder	60
ECE 5	negative	Non-pathogenic	Cloaca		Broiler breeder	20
ECE 6	positive	Non-pathogenic	Cloaca		Broiler breeder	17
ECE 7	negative	Pathogenic	Bone marrow of femur	osteomyelitis	Broiler	3

[1]Strain classifications based on the origin of the sample, *i.e.* positive samples isolated from macroscopic lesions in broilers were classified as "pathogenic", samples originated from cloaca of apparently healthy birds were classified "non-pathogenic".

[2]Negative control group, all inoculations were performed blindly and groups were randomly assigned.

***Enterococcus. cecorum* strains.** Prior to the pilot, a set of 28 different *E. cecorum* strains was created for research purposes. The set consisted of 14 strains isolated from cloacal swabs collected from clinical healthy broiler breeder chickens and 14 strains isolated from typical *E. cecorum* lesions such as pericarditis, arthritis, osteomyelitis, spondylolisthesis and necrosis of the femur head in broilers. From this database a selection of 6 different *E. cecorum* strains (table 1) was made to perform the pilot. Two strains are considered pathogenic, they were collected from lesions. The other 4 strains were collected from cloacal swabs, thus considered non-pathogenic.

Inoculum. A day prior to the inoculation, the *E. cecorum* strains were recovered from cryopreservation (-70 degrees Celsius), transferred to sheep blood agar plates (10%) and incubated during 24 hours at 37°C with 5% CO_2. Bacterial colonies were scraped from the blood agar plates and diluted into peptone physiological saline solution (PPS) to match the turbidity of 0.5 McFarland standard (10^8 CFU/ml) using a Biosan DEN-1 McFarland Densitometer. The 0.5 McFarland solution was serial diluted to a concentration of 10^3 CFU/ml, of this concentration 0.1 ml per egg was used for inoculation, i.e. 10^2 CFU/egg. In order to count the CFU/ml, a series of dilutions of 10^4 CFU/ml, 10^3 CFU/ml and 10^2 CFU/ml were incubated for 24 hours at 37°C with 5% CO2 and colony forming units were counted. After the preparations of the inoculums, the inoculums were preserved and transported on ice. In addition to the 6 *E. cecorum* strains, a control group (ECE 3) was added during the inoculum preparation. This control group inoculum contained 0.9% PPS.

Pre-inoculation. Groups of 5 eggs were candled and the air pockets were marked prior to placement onto a cardboard egg tray. Subsequently, the eggs were decontaminated with 99.8% isopropanol and placed into the laminar flow cabinet. The remaining of the inoculation process was performed in the laminar flow cabin in order to minimize contamination of the embryonated egg. Each egg was marked and the final preparation was decontaminating of the inoculation site with cotton wool drenched into a 7% iodine tincture. Using a decontaminated engraver (Hugo Brennenstuhl GmbH & Co Kommanditgesellschaft, Electric Engraver Signograph 25 Set, article number 1500740, Tübingen, Germany), the eggshells were removed at the inoculation sites.

Post inoculation. All inoculated eggs were candled every 24 hours up to 8 days post inoculation or until the end of the pilot, i.e. incubation day 18. If there was any doubt whether the embryo was still alive, a second opinion determined the decisive choice.

Inoculation procedures. Systematic approaches for each of the inoculation routes were defined according to the manual for viral inoculation[17], previously mentioned literature and our own experiences. The inoculations were performed using a syringe with different sizes and lengths of needles and have been performed as follows.

Amniotic/embryonic inoculation. In the centre of the blunt end of the egg a hole was made in the shell. The full length of a 1 or 1.5 inch 21G needle was injected following the central axis of the egg.

Allantoic cavity inoculation. The air chamber was marked at the lowest point and 3-4mm above this point the eggshell was penetrated and the full length of a 5/8 inch, 21-gauge needle was injected parallel on the central axis of the egg.

Chorion-allantoic-membrane inoculation.
An artificial air chamber on top of the CAM was created as follows: a hole in the egg shell and shell membrane at the blunt egg of the egg was made. Halfway the length of the egg, between two major blood vessels a hole was made solely in the egg shell. The shell membranes were gently penetrated with a blunt needle. With a squeezed rubber pipette bulb, placed on the hole at het blunt end of the egg, negative pressure was created inside the egg. The negative pressure causes the chorion-allantoic membrane to release from the inside of the shell membrane which forms the artificial air chamber in which the inoculum was injected, using a 21-gauge, 5/8 inch needle.

Yolk sac inoculation. After marking the air chamber, a hole in the centre of the chamber was made. Following the central axis of the egg to a point just beyond the centre of the egg, a 1.5 inch 21-gauge needle was injected to inoculate into the yolk sac.

Intravenous inoculation. While candling the egg, a large blood vessel was marked with a pencil, a triangle with sides of approximately 4 mm on top of the vessel was removed in the egg shell. The sites were penetrated with the engraver and the eggshell was carefully removed with a blunt needle and subsequently 0,5 inch, 32-gauge needles were used for inoculation.

Statistical analysis
Using the Cox proportional hazards model with RStudio[20], the equalities of the survival distributions of the different inoculations groups were determined

(appendix 1). The p-value of equality was set at a significance level of 0,05, thus p-values below 0,05 were considered as significant differences. Due to a limited amount of statistical power and to prevent an underpowered statistical analysis, the age of the embryos was not considered in the statistical analyses.

Results

Part I – Success rates of inoculation

Amniotic/embryonic inoculation. Results are shown in table 2, only 14 day-old embryos were inoculated with a relatively high success-rate (95%) while on the other egg incubation days the success-rates varied between 35% (embryonic day 10) and 85% (embryonic day 16). At no incubation day, a 100% score was obtained, therefore, the AE route was not used in the second part of the pilot.

Table 2. Success rates of the inoculation of amniotic cavity/embryo with methylene blue using 1.5 inch needles. Per incubation day 20 embryonated eggs were used.

Incubation day	Success-rates AE inoculation
10	35%
12	70%
14	95%
16	85%
17	70%
18	80%

Yolk sac inoculation. The yolk sac inoculations were performed with a 25% success-rate in the 10 days and in the 14 days incubated eggs. Therefore, the decision was made to not use the YS in the second part.

Intravenous inoculation. The IV inoculation was performed with a 100% success-rate for both the 10 and 14 days incubated eggs. Due to the high success rate the decision was made to test the IV route in the ELA of part II.

Table 3.CFU/ml per strain determined for incubation day 10 and 14.

	CFU/ml	
	Incubation day	
Strain	10	14
ECE 1	65	86
ECE 2	5	49
ECE 3	NEG	NEG
ECE 4	5	101
ECE 5	101	101
ECE 6	4	12
ECE 7	79	91

Part II– Embryo lethality assay .

Inoculum plate count. Table 3 shows the number of colonial-forming units/ml (CFU). The target was to have 100CFU/ml in every inoculum except ECE 3, as it was the negative control group.

Survival AC inoculation. In total 280 embryonated eggs were inoculated using the AC inoculation route and 189 embryos survived until the end of the experiment (Table 4). The average survival percentage for eggs with incubation day 10 was 67% and 73% for the eggs inoculated at 14 days of incubation. A significant difference was determined between ECE 2 and the other strains except for ECE 3 (p-value 0,11).

Table 4. Survival rates results of the embryo lethality assay for the allantoic cavity (AC), chorion-amnion-membrane (CAM) and intravenous inoculation (IV) arranged by incubation day.
^{AB} Groups with different letters within a column are significantly different (P<0.05)

	Survival Rate					
	Route of inoculation					
	AC		CAM		IV	
	Incubation day		Incubation day		Incubation day	
Strain	**10**	**14**	**10**	**14**	**10**	**14**
ECE 1	$15/20^{A}$	$16/20^{A}$	$7/20^{A}$	$8/19^{A}$	$3/11^{A}$	-
ECE 2	$6/20^{B}$	$10/20^{B}$	$4/20^{B}$	$4/16^{B}$	$4/9^{B}$	-
ECE 3	$10/20^{AB}$	$15/20^{AB}$	$14/20^{AB}$	$14/20^{AB}$	$10/11^{AB}$	10/11
ECE 4	$14/20^{A}$	$16/20^{A}$	-	-	-	-
ECE 5	$11/20^{A}$	$17/20^{A}$	-	-	-	-
ECE 6	$16/20^{A}$	$14/20^{A}$	-	-	-	-
ECE 7	$13/20^{A}$	$16/20^{A}$	-	-	-	-

Survival CAM inoculation. In total 115 embryos were successfully inoculated using the CAM inoculation route and 51 survived the experiment (Table 4). The average embryonic survival for the eggs inoculated at incubation days 10 and 14 was 42% and 46% respectively . The lowest survival, only 20%, occurred with ECE 2. The strains were all significantly different (P<0.05).

Survival IV inoculation. Of the 42 embryos that were successfully inoculated, only 35% of the embryos survived (table 4). Statistical analyses determined that the survival of the ECE 1 strain was significantly lower in comparison with the ECE 3 control group.

Embryonic survival curves. The embryonic survival curves are visible in figure 1-5 for each inoculation route and egg incubation day. In the group inoculated with the ECE 2 strain, a potential pathogenic strain, the

survival rate decreased during a longer period of time than the other strains. The pathogenic ECE 2 and ECE 7 only have a mean survivability of 56% compared to the non-pathogenic strains (ECE 1, ECE 4, ECE 5 and ECE 6) of 74.5%.

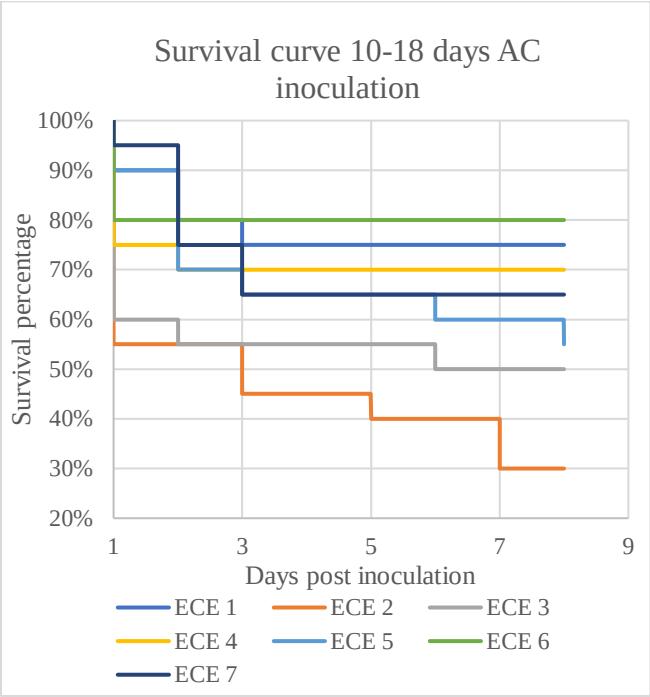

Figure 1. Survival curves for the AC inoculated eggs (10 days incubated) with the E. cecorum strains and the control PPS (ECE 3).

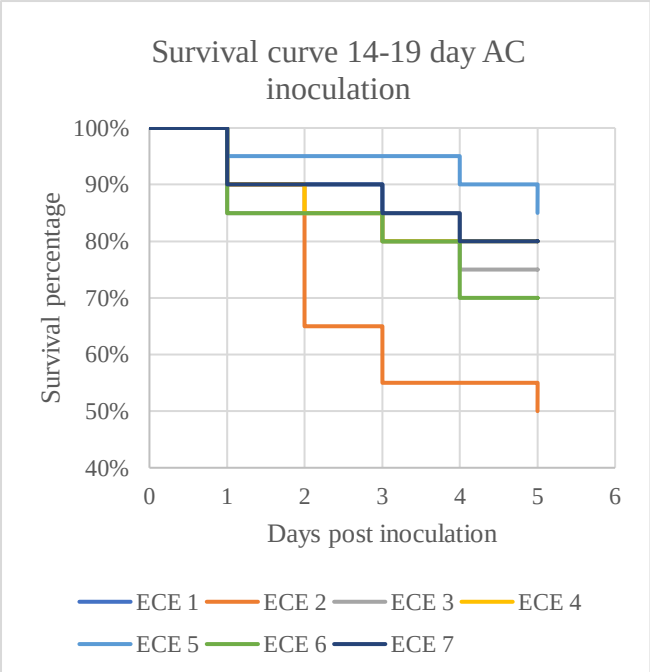

Figure 2. Survival curves for the 14 day-incubated eggs that were AC inoculated with the E. cecorum strains and the control (ECE 3).

The survival curves of the CAM inoculated embryonated eggs show similarities after the AC inoculated embryonated eggs. Especially in regards to the ECE 2 strain. This strain again continued to kill for a longer period of time and it eventually has the lowest survival rate. The pathogenic strain only had a survivability of 22% after CAM inoculation while the non-pathogenic and the control group have a survival rate of respectively 38.5% and 70%. However, this does not correspond with the AC inoculation where the non-pathogenic strains had a higher survival rate than the control group.

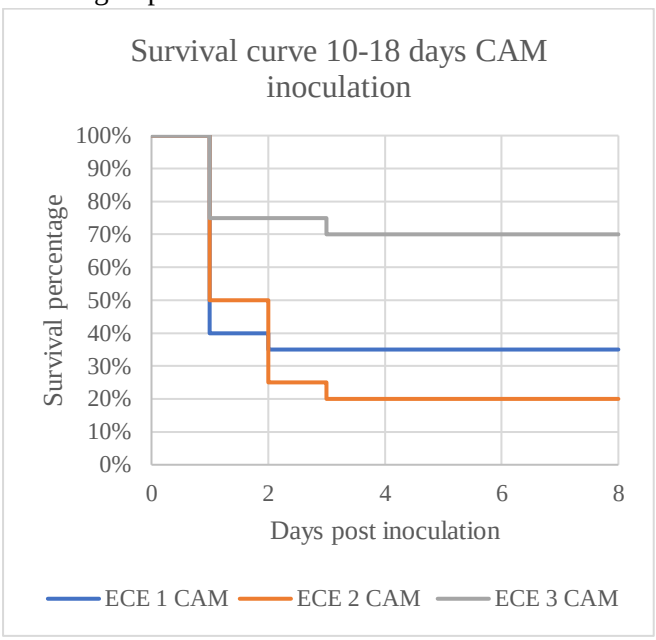

Figure 3. Survival curves for the 10 day-old eggs inoculated using the chorion-allantois-membrane inoculation route.

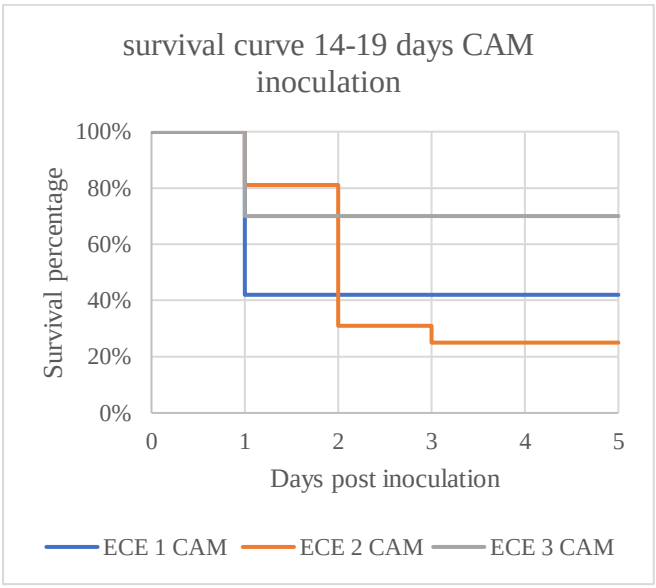

Figure 4. Survival curves for the 14 day-incubated eggs with the chorion-allantois-membrane inoculation route. Similar to inoculation of the 10 day-old embryos, death only occurs in the first 3 days and the pathogenic ECE 2 strain kills the most embryos.

The embryonic survival rates of IV inoculation show little similarities with the AC and CAM inoculation. The non-pathogenic ECE 1 strain only has a survivability of 27%, while the pathogenic strain has a survival rate of 44%. Although the IV inoculations were

performed with fewer embryonated eggs, it does show that the IV inoculated ECE 3 control group has a much higher survival rate in comparison with the other 2 routes of inoculation: 95% of the embryos survived the inoculation with PPS.

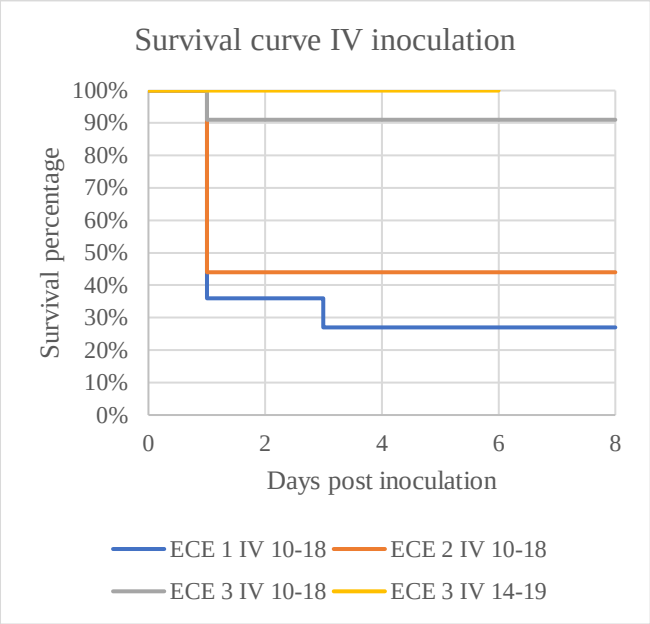

Figure 5. Survival curves for the 10 and 14 day-old eggs inoculated intravenously. The results are not comparable with either the allantoic cavity or chorion-allantois-membrane inoculation route.

Discussion

In these two experiments the pathogenicity of 6 different *E. cecorum* strains were compared using an embryo lethality assay (ELA). In the first experiment the success-rates of inoculation were very variable. The experiment included the AE inoculation route, which is not commonly used at embryonic ages younger than 17 days. From day 17 on, *in ovo* vaccinations against Marek's disease targeting the amniotic cavity or the embryo achieved success rates of 97% in the AE inoculation[21]. The success rates of AE inoculation, prior to incubation day 17, can be improved by removal of the top of the blunt side of the egg[17]. By doing this latter the amniotic cavity is within sight, disadvantage of this technique is it will increased the chance of bacterial contamination and the technique is cumbersome.

Based on our results from the AC inoculation, ECE 2 is the most pathogenic strain. In the ECE 2 strain inoculated egg the survival rate decreased during a longer period of time compared to eggs inoculated with other strains (figure 1 and 2). Strain ECE 2 was isolated from typically lesion, i.e. osteomyelitis in the 6th vertebrae, therefore we expect ECE 2 to be classified as a pathogenic strain. The survival rate of inoculated eggs with strain ECE 7, a potential pathogenic strain, after AC inoculation did not significant differ with the control group and the eggs inoculated with non-pathogenic strains. Therefore, according to this ELA this specific strain could be classified as non-pathogenic despite it was isolated from a typical *E. cecorum* lesion.

The control group survival rates are also peculiar. Of the 102 successfully sham inoculated embryonated eggs only 73 embryos survived (table 4). Especially in the AC inoculated eggs, the mean survival of 72%, is less than the expected survival rate between 100% and 93%, which has been found in other ELA[11,13]. However, the bacterial plate count yielded no growth and no macroscopic lesions were observed during the inspections of the embryos of the control group, which could have explained the number of deaths occurring due to a possible contamination of the inocula or manual inoculation errors. Thereafter, the IV inoculation with ECE 3 did not influence the survival rate, were only a single embryo died. No clear explanations were found for the decreased survival rates of the AC inoculated embryonated eggs.

IV inoculation were performed during candling of the eggs, the inoculations were visible and unsuccessful inoculations could be discarded. Therefore, it is possible to achieve inoculations with a 100% success rate. IV inoculations are cumbersome and success of inoculation depends heavily on the experience of the inoculator. Subsequently, minor aberration during or after inoculation might cause (severe) bleeding. In this experiment the survival rates after IV inoculation did not show a significant difference between the non-pathogenic ECE 1 and the pathogenic ECE 2 strain. Either, this inoculation route is not appropriate for an ELA or better defined ECE strains and/or more embryonated eggs need to be incorporate to detect a difference in the survival rates of different strains.

Significant differences in the survival rates of the CAM inoculated groups were observed. A limited number of groups (n=3) were used, due to the limited amount of eggs available, the 4 remaining ECE strains were not inoculated onto the CAM. Future studies may provide further insights if embryo survival rates after CAM inoculations are suitable for biotyping *E. cecorum*.

Although the inocula were prepared under guidance off a senior lab technician and in accordance with standard procedures, a variance in concentration (CFU/ml) in the different inocula became apparent during the bacterial plate counts. The CFU counts were lower than the expected and goal of 100 CFU/ml. We hypothesised that a delayed bacterial growth was a possible explanation for the lower concentration. Therefore the blood agar plates were incubated for an additional 24 hours. The count of CFU increased after the additional incubation, this latter suggest that the CFU/ml of the

inoculum was sufficient (data not shown). This latter was also observed in other studies were the phenotypical and genotypical characterization of *E. cecorum* strains were examined, differences in growth between strains was observed.[23]. During previous performed ELA[13] variations in CFU/ml occurred and it did not influence the results negatively.

Conclusion

Based on our results, the CAM inoculation technique is currently the best technique to discriminate between *E. cecorum* strains in an embryo lethality assay. Thereafter, the AC technique provided evidence that pathogenic strains can be distinguished from a non-pathogenic strains. The IV inoculation route has the potential to be useful, future research is needed to provided evidence of the utility in embryo lethality assays. Based on this pilot, an ELA with AC and CAM inoculation route provides reproducible and reliable methods to discriminate *E. cecorum* strains and is suitable for future *E. cecorum* related research.

1. Shane SM, Stern NJ. *Diseases of Poultry*.; 2003. doi:10.1354/vp.41-4-450
2. Jung A, Chen LRM, Suyemoto M, Barnes JH, Borst LB. A Review of Enterococcus cecorum Infection in Poultry. *Avian Dis*. 2018;In-Press(In-Press):In-Press. doi:10.1637/11825-030618-Review.1
3. Devriese LA, Dutta GN, Farrow JAE, Van de Kerkchoven A, Philips BA. Streptococcus cecorum, a new species isolated from chickens. *Int J Syst Evol Microbiol*. 1983;(33):772-776.
4. Jung A, Rautenschlein S. Comprehensive report of an Enterococcus cecorum infection in a broiler flock in Northern Germany. *BMC Vet Res*. 2014. doi:10.1186/s12917-014-0311-7
5. Borst LB, Suyemoto MM, Keelara S, Dunningan SE, Guy JS, Barnes HJ. A Chicken Embryo Lethality Assay for Pathogenic *Enterococcus cecorum*. *Avian Dis*. 2014;58(2):244-248. doi:10.1637/10687-101113-Reg.1
6. Borst LB, Suyemoto MM, Sarsour AH, et al. Pathogenesis of Enterococcal Spondylitis Caused by Enterococcus cecorum in Broiler Chickens. *Vet Pathol*. 2017. doi:10.1177/0300985816658098
7. De Herdt P, Defoort P, Van Steelant J, et al. Enterococcus cecorum osteomyelitis and arthritis in broiler chickens. *Vlaams Diergeneeskd Tijdschr*. 2009.
8. Stalker MJ, Brash ML, Weisz A, Ouckama RM, Slavic D. Arthritis and osteomyelitis associated with Enterococcus cecorum infection in broiler and broiler breeder chickens in Ontario, Canada. *J Vet Diagnostic Investig*. 2010. doi:10.1177/104063871002200426
9. Borst LB, Suyemoto MM, Scholl EH, Fuller FJ, Barnes HJ. Comparative genomic analysis identifies divergent genomic features of pathogenic Enterococcus cecorum including a type IC CRISPR-cas system, a capsule locus, an epa-like locus, and putative host tissue binding proteins. *PLoS One*. 2015. doi:10.1371/journal.pone.0121294
10. Dolka B, Chrobak-Chmiel D, Czopowicz M, Szeleszczuk P. Characterization of pathogenic Enterococcus cecorum from different poultry groups: Broiler chickens, layers, turkeys, and waterfowl. *PLoS One*. 2017. doi:10.1371/journal.pone.0185199
11. Wooley RE, Gibbs PS, Brown TP, Maurerb J. Chicken Embryo Lethality Assay for Determining the Virulence of Avian Escherichia coli Isolates. *Avian Dis*. 2000. doi:10.1080/03079457.2013.816654
12. Seo HS, Cha SY, Kang M, Jang HK. Chicken embryo lethality assay for determining the virulence of Riemerella anatipestifer isolates. *Avian Pathol*. 2013;42(4):387-392. doi:10.1080/03079457.2013.816654
13. Blanco AE, Barz M, Icken W, et al. Chicken embryo lethality assay for determining the lethal dose and virulence of Enterococcus faecalis. *Avian Pathol*. 2017;46(5):548-555. doi:10.1080/03079457.2017.1324942
14. Powell CJ, Finkelstein RA, Nolan LK, et al. The In Ovo Chick Chorioallantoic Membrane (CAM) Assay as an Efficient Xenograft Model of Hepatocellular Carcinoma. *J Bacteriol*. 2013. doi:10.2307/1589501
15. Powell CJ, Finkelstein RA. Virulence of Escherichia coli strains for chick embryos. *J Bacteriol*. 1966;91(4):1410-1417. doi:10.2307/1589501
16. Peers BYJH, Ransom SE, Huebner ARJ. The pathologic changes produced in chick embryos by yolk sac inoculation of group A coxsackie virus (from the Federal Security Agency , Public Health Service , National Institutes of Health , Bethesda). 1952.
17. Venter E. Applied Veterinary Virology : The isolation and identification of viruses using cell

cultures. 2009:1-5.

18. Kelling CL, Schipper IA. Simplified method for efficient intravascular inoculation of chicken embryos. *J Clin Microbiol*. 1976;4(1):104-105.

19. Seto F. Early development of the avian immune system. *Poult Sci*. 1981;60(9):1981-1995. doi:10.3382/ps.0601981

20. RStudio. RStudio Team (2019).

21. Wakenell PS, Bryan T, Schaeffer J, Avakian A, Williams C, Whitfill C. Effect of in ovo vaccine delivery route on herpesvirus of turkeys/SB-1 efficacy and viremia. *Avian Dis*. 2002. doi:10.1637/0005-2086(2002)046[0274:EOIOVD]2.0.CO;2

22. Seo HS, Cha SY, Kang M, Jang HK. Chicken embryo lethality assay for determining the virulence of Riemerella anatipestifer isolates. *Avian Pathol*. 2013. doi:10.1080/03079457.2013.816654

23. Dolka B, Chrobak-Chmiel D, Makrai L, Szeleszczuk P. Phenotypic and genotypic characterization of Enterococcus cecorum strains associated with infections in poultry. *BMC Vet Res*. 2016. doi:10.1186/s12917-016-0761-1

Appendix 1

These Tables show the statistical analysis of each ECE group. When the P-value (Pr (>|z|)) is below 0,05, there is a marginal significance difference between the ECE groups.

Injection routes and strains statistically compared to ECE 1	Coef	Exp(coef)	se(coef)	Z	Pr (>|z|)
AC, ECE 2	1.20425	3.33426	0.39119	3.078	0.002081
AC, ECE 3	0.67946	1.97282	0.42177	1.611	0.107188
AC, ECE 4	0.16642	1.18107	0.45949	0.362	0.717211
AC, ECE 5	0.29352	1.34113	0.44098	0.666	0.505672
AC, ECE 6	0.14070	1.15108	0.45948	0.306	0.759442
AC, ECE 7	0.20545	1.22808	0.44948	0.457	0.647601
CAM, ECE 2	-1.11638	0.32746	0.47991	-2.326	0.020008
CAM, ECE 3	-1.83062	0.16031	0.55058	-3.325	0.000884
IV, ECE 2	-1.61246	0.19940	0.69171	-2.331	0.019746
IV, ECE 3	-2.70047	0.06717	1.14255	-2.364	0.018101
inoc <- relevel(factor(inoculation.strain),"ECE1") fit.cox3 <- coxph (Surv(age,dead)~factor(injection.route)+factor(age.at.inoculation)+inoc:factor(injection.route))					

Injection routes and strains statistically compared to ECE 2	Coef	Exp(coef)	se(coef)	Z	Pr (>|z|)
AC, ECE 1	-1.20425	0.29992	0.39119	-3.078	0.002081
AC, ECE 3	-0.52479	0.59168	0.32921	-1.594	0.110922
AC, ECE 4	-1.03783	0.35422	0.37661	-2.756	0.005856
AC, ECE 5	-0.91073	0.40223	0.35377	-2.574	0.010043
AC, ECE 6	-1.06355	0.34523	0.37676	-2.823	0.004759
AC, ECE 7	-0.99880	0.36832	0.36438	-2.741	0.006123
CAM, ECE 1	-0.08787	0.91588	0.27922	-0.315	0.752978
CAM, ECE 3	-1.23904	0.28966	0.34621	-3.579	0.000345
IV, ECE 1	0.40821	1.50412	0.57021	0.716	0.474060
IV, ECE 3	-1.61280	0.19933	1.09724	-1.470	0.141598
inoc <- relevel(factor(inoculation.strain),"ECE2") fit.cox3 <- coxph (Surv(age,dead)~factor(injection.route)+factor(age.at.inoculation)+inoc:factor(injection.route))					

Injection routes and strains statistically compared to ECE 3	Coef	Exp(coef)	se(coef)	Z	Pr (>|z|)
AC, ECE 1	-0.6795	0.5069	0.4218 -	1.611	0.107188
AC, ECE 2	0.5248	1.6901	0.3292	1.594	0.110922
AC, ECE 4	-0.5130	0.5987	0.4083	-1.257	0.208931
AC, ECE 5	-0.3859	0.6798	0.3874	-0.996	0.319100
AC, ECE 6	-0.5388	0.5835	0.4084	-1.319	0.187099
AC, ECE 7	-0.4740	0.6225	0.3971	-1.194	0.232584
CAM, ECE 1	1.1512	3.1619	0.3539	3.253	0.001143
CAM, ECE 2	1.2390	3.4523	0.3462	3.579	0.000345
IV, ECE 1	2.0210	7.5459	1.0621	1.903	0.057054
IV, ECE 2	1.6128	5.0168	1.0972	1.470	0.141598

inoc <- relevel(factor(inoculation.strain),"ECE3")
fit.cox3 <- coxph (Surv(age,dead)~factor(injection.route)+factor(age.at.inoculation)+inoc:factor(injection.route))

Strains statistically compared to ECE 4	Coef	Exp(coef)	se(coef)	Z	Pr (>|z|)
AC, ECE 1	-0.16642	0.84669	0.45949	-0.362	0.717211
AC, ECE 2	1.03783	2.82308	0.37661	2.756	0.005856
AC, ECE 3	0.51304	1.67036	0.40831	1.257	0.208931
AC, ECE 5	0.12709	1.13552	0.42819	0.297	0.766608
AC, ECE 6	-0.02572	0.97460	0.44724	-0.058	0.954133
AC, ECE 7	0.03903	1.03980	0.43696	0.089	0.928822
inoc <- relevel(factor(inoculation.strain),"ECE4") fit.cox3 <- coxph (Surv(age,dead)~factor(injection.route)+factor(age.at.inoculation)+inoc:factor(injection.route))					

Strains statistically compared to ECE 5	Coef	Exp(coef)	se(coef)	Z	Pr (>|z|)
AC, ECE 1	-0.29352	0.74564	0.44098	-0.666	0.505672
AC, ECE 2	0.91073	2.48615	0.35377	2.574	0.010043
AC, ECE 3	0.38595	1.47101	0.38738	0.996	0.319100
AC, ECE 4	-0.12709	0.88065	0.42819	-0.297	0.766608
AC, ECE 6	-0.15282	0.85829	0.42823	-0.357	0.721195
AC, ECE 7	-0.08806	0.91570	0.41744	-0.211	0.832921
inoc <- relevel(factor(inoculation.strain),"ECE5") fit.cox3 <- coxph (Surv(age,dead)~factor(injection.route)+factor(age.at.inoculation)+inoc:factor(injection.route))					

Strains statistically compared to ECE 6	Coef	Exp(coef)	se(coef)	Z	Pr (>|z|)
AC, ECE 1	0.14070	0.86875	0.45948	-0.306 0	.759442
AC, ECE 2	1.06355	2.89664	0.37676	2.823	0.004759
AC, ECE 3	0.53876	1.71389	0.40840	1.319	0.187099
AC, ECE 4	0.02572	1.02606	0.44724	0.058	0.954133
AC, ECE 6	0.15282	1.16511	0.42823	0.357	0.721195
AC, ECE 7	0.06476	1.06690	0.43697	0.148	0.882188
inoc <- relevel(factor(inoculation.strain),"ECE6") fit.cox3 <- coxph (Surv(age,dead)~factor(injection.route)+factor(age.at.inoculation)+inoc:factor(injection.route))					

Strains statistically compared to ECE 7	Coef	Exp(coef)	se(coef)	Z	Pr (>|z|)
AC, ECE 1	-0.20545	0.81428	0.44948	-0.457	0.647601
AC, ECE 2	0.99880	2.71501	0.36438	2.741	0.006123
AC, ECE 3	0.47401	1.60642	0.39708	1.194	0.232584
AC, ECE 4	-0.03903	0.96172	0.43696	-0.089	0.928822
AC, ECE 5	0.08806	1.09205	0.41744	0.211	0.832921
AC, ECE 6	-0.06476	0.93730	0.43697	-0.148	0.882188
inoc <- relevel(factor(inoculation.strain),"ECE7") fit.cox3 <- coxph (Surv(age,dead)~factor(injection.route)+factor(age.at.inoculation)+inoc:factor(injection.route))					