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RESEARCH ARTICLE

The Role of Enterococcal Virulence Factors on Experimental Amyloid Arthropathy in Chickens [1]

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Summary

In this study, it was aimed to determine the role of enterococcal virulence factors on the development of amyloid arthropathy in chickens. *Enterococcus faecalis* strains phenotypically variable in gelatinase, aggregation substance and cytolisine traits were inoculated intraarticularly to chickens of two weeks and intravenously to chickens of five weeks. In intraarticular inoculation groups, amyloid arthropathy occurred in 65.2% of gelatinase positive strains, 30.4% of aggregation substance positive strains and 22.2% of cytolisine positive strains. In intravenous inoculation groups, bilateral amyloid arthropathy occurred in 75% of gelatinase positive strains, 50% of aggregation substance positive strains and 26.6% of cytolisine positive strains. Gelatinase was the only virulence factor common in all *E. faecalis* strains causing amyloid arthropathy, and none of the gelatinase negative strains caused the amyloid arthropathy in any of the chickens in both groups. In conclusion; it was suggested that enterococcal gelatinase plays a role in the development of experimental amyloid arthropathy and the gelatinase positive *E. faecalis* strains may cause amyloid arthropathy which is characterized by arthritis, lameness and growth retardation.

Keywords: *Amyloid arthropathy, Chicken, Enterococcus faecalis, Virulence*

Tavukların Deneysel Amiloid Artropatisinde Enterokokal Virulens Faktörlerinin Rolü

Özet

Bu araştırmada, enterokokal virulens faktörlerinin tavukların amiloid artropatisinin oluşumu üzerindeki etkilerinin araştırılması amaçlandı. Çalışmada jelatinaz, agregasyon maddesi ve sitolizin yönünden çeşitlilik gösteren *E. faecalis* suşları 2 haftalık tavuklara intraartiküler ve 5 haftalık tavuklara intravenöz yollar ile inokule edildi. İntraartiküler deney gruplarında jelatinaz pozitif suşlar inokule edilen gruplarda %65.2, agregasyon maddesi pozitif suşlar verilen gruplarda %30.4 ve sitolizin pozitif suşlar verilen gruplarda %22.2 oranında amiloid artropati saptandı. İntravenöz deney gruplarında jelatinaz pozitif suşlar inokule edilen gruplarda %75, agregasyon maddesi pozitif suşlar verilen gruplarda %50 ve sitolizin pozitif suşlar verilen gruplarda %26.6 oranında bilateral amiloid artropati saptandı. Her iki deney grubunda da amiloid artropatiye neden olan suşlarda ortak olan tek virulens faktörünün jelatinaz olduğu belirlenirken, jelatinaz taşımayan suşların hiçbirisi amiloid artropatiye neden olmadı. Sonuç olarak, enterokokal jelatinazın tavuklarda deneysel amiloid artropati oluşumunda rol oynadığı ve jelatinaz pozitif *E. faecalis* suşlarının tavuklarda artritis, topallık ve büyüme geriliği ile karakterize amiloid artropatiye neden olabileceği kanısına varıldı.

Anahtar sözcükler: *Amiloid artropati, Enterococcus faecalis, Tavuk, Virulens*

INTRODUCTION

Enterococcus spp. which are found commensally in the oral cavity, gastrointestinal tract, genital tract, urinary tract and on the skin of various animals and humans [1,2] cause nosocomial bacteriemia, endocarditis, peritonitis, urinary tract infections, soft tissue infections, subdural empyema, osteomyelitis and endophtalmitis in humans [3,4]. *Enterococcus* spp. have been reported to isolated from liver abscesses, discospondylitis, septicemia, lower respiratory diseases and urinary tract infections in dogs [5-9] from cholangiohepatitis,

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enteritis, pancreatitis and cholangitis in cats [10,11]. *E. faecalis* and *E. faecium* are the most common etiological agents of enterococcal infections [12]. The studies on enterococci have increased in parallel with elucidation of the significance of enterococcal infections in humans and of the role of the animals in transmission of vancomycine resistant enterococci to humans. One of the most important enterococcal infections in poultry is amyloid arthropathy. In Galliformes, amyloid accumulation in joints was first isolated by Maestrini and Pascuci from guinea fowl *(Numida meleagris galeata)* in 1970 and they observed that it was characterized by growth retardation and lameness in 5 percent. In commercial turkey flocks *(Meleagris gallopavo gallopavo)*, amyloid accumulation has also seen in synovia as well as in internal organs [13]. Amyloid arthropathy in brown layer chickens was first reported and characterized by growth retardation and lameness as clinical symptoms [14]. Amyloid arthropathy has recently become a clinical problem which affected about 20-30% of European chicken flocks [15].

Several virulence factors contributing to enterococcal infections have been described: aggregation substance (AS), cytolisine, gelatinase, lipoteichoic acid, hyaluronidase, AS-48, lipase, hemagglutinin, surface carbohydrates, extracellular surface proteins, bacteriocin and extracellular superoxide dismutase (SOD) [16,17]. Among them, virulence factors studied most intensively are cytolisine, AS and gelatinase.

The elicitation of the roles of virulence factors in amyloid arthropathy caused by *Enterococcus faecalis* was aimed in this study.

MATERIAL and METHODS

Strains

Arthropathic and amyloidogenic *E. faecalis* 6085.94 strain was obtained from Dr. W.J.M. Landman (Poultry Health Center, Doorn, Netherlands). *E. faecalis* OG1X strain and five strains including isogenic variants for virulence factors were provided by the culture collection of University of Ankara, Faculty of Veterinary Medicine, Department of Microbiology (originated from Dr. D.B.Clewell, Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, Michigan, USA). Three *E. faecalis* strains isolated from chickens with arthritis examined in the Microbiology Laboratory of Veterinary Faculty in University of Ankara were also used. All the strains were stored at -20°C and -80°C until they were used.

The phenotypic and biochemical properties were examined to identify strains isolated from chicken with arthritis and to confirm the other reference enterococcus strains. Bacteria showing the yellow color in Enterococcus Presumptive broth and brown-black colonies on D-coccocell agar were suspected as enterococci. These strains examined for Gram reaction, catalase reaction, growing at 45°C, fermentation of sorbose, arabinose, raffinose, mannitol, sorbitol and sucrose, motility, telluride tolerance and yellow pigmentation and identified at species level [3].

Detection of Virulence Factors

Aggregation substance (AS): Aggregation substance production of *E. faecalis* strains were examined by clumping assay [18]. This assay was performed on the strains isolated from chickens with artritis and all OG1X strains. In this assay, *E. faecalis* OG1X (pAM714) strain was used as a positive control, *E. faecalis* OG1X (pAM944) strain was used as a negative control. Strains showing aggregation were considered as AS positive, strains not forming aggregation were considered as AS negative.

Cytolisine: Cytolisine activity was tested as described [19]. *E. faecalis* OG1X (pAM714) strain was used as a positive control, *E. faecalis* OG1X (pAM9058) strain was used as a negative control in this assay. Colonies surrounded by beta hemolytic zone were identified as cytolisine positive.

Gelatinase: Gelatinase activity was examined as described [20]. In the gelatinase assay, *E. faecalis* OG1RF strain was used as a positive control, *E. faecalis* OG1X (pAM714) strain was used as a negative control. After the medium which was gelatinous normally were kept at 4°C, the observation of aqueous formation was considered as positive for gelatinase activity.

Experimental Infection

Experimental Animals: One day brown layer chicks (fifty-four) and 3 weeks- old chickens (forty-five) were provided by a commercial chicken enterprise. Animals were kept for 2 weeks for adaptation. Animals were fed ad libitum during adaptation period.

Preparation of the inoculums: Inoculation from stock culture of strains was done onto Brain Heart Infusion (BHI) agar containing blood to prepare the inoculums for experimental infection. At the end of incubation period, colonies were passaged to Brain

Hearth Infusion (BHI) broth containing 10% sera and were incubated at 37°C for 24-48 h. After incubation period, cultures were adjusted to two different concentration, 10^7 CFU/ml and 10^9 CFU/ml, using BHI broth according to McFarland nephelometer standards.

Experimental groups and experimental inoculations: After the adaptation period, 9 experimental groups included six 2 week old chickens and 9 groups included five 5 week old chickens were designed.

Intraarticular inoculation groups: Nine experimental groups composed of six 2 week old chickens were inoculated with 0.1 ml of 10^7 CFU/ml enterococcus strains mentioned below by intraarticular route.

A group named as Group 1A was inoculated with *E. faecalis* 6085.94 known as arthropathic and amyloidogenic and this group was used as positive control. Group 1B was inoculated with *E. faecalis* OG1RF strain (Gelatinase positive), Group 1C was inoculated with *E. faecalis* OG1X(pAM9058) strain (AS positive), Group 1D was inoculated with *E. faecalis* OG1SSP strain (Gelatinase and AS positive), Group 1E was inoculated with *E. faecalis* OG1X(pAM714) strain (Cytolisine and AS positive), Group 1F was inoculated with *E. faecalis* isolate 1 strain (Cytolisine positive), Group 1G was inoculated with *E. faecalis* isolate2 strain (Gelatinase, AS and Cytolisine positive) and Group 1H was inoculated with *E. faecalis* isolate3 strain (Gelatinase, AS and Cytolisine negative). Group 1I was inoculated with 0.1 ml BHI broth (containing 10% serum) and this group was used as negative control.

Intravenous Inoculation Groups: Nine groups included five 5 week old chickens were inoculated with 0.1 ml of 10^9 CFU/ml enterococcus strain mentioned below intravenously.

Group 2A was designed as positive control and was inoculated with arthropathic and amyloidogenic *E. faecalis* 6085.94 strain. Group 2B was inoculated with *E. faecalis* OG1RF strain (Gelatinase positive), Group 2C was inoculated with *E. faecalis* OG1X(pAM9058) strain (AS positive), Group 2D was inoculated with *E. faecalis* OG1SSP strain (Gelatinase and AS positive), Group 2E was inoculated with *E. faecalis* OG1X(pAM714) strain (Cytolisine and AS positive), Group 2G was inoculated with *E. faecalis* isolate1strain (Cytolisine positive), Group 2G was inoculated with *E. faecalis* isolate2 strain (Gelatinase, AS and Cytolisine positive) and Group 2H was inoculated with *E. faecalis* isolate3 strain (Gelatinase, AS and Cytolisine negative). Group 2I was used as negative control and was inoculated with 01 ml BHI broth (containing 10% serum).

Post-inoculation Examinations

Evaluation of Clinical and Pathologic Results: Animals in intraarticular experimental group were observed for 7 weeks and animals in intravenous experimental group were observed for 10 weeks after inoculation. Animals were examined for arthritis during these periods. Daily weight gains of these animals were recorded before and after experiments. Animals dead in observation period and killed by cervical dislocation at the end of this period were examined by necropsy and were examined especially for pathological symptoms microscopically in joints. Accumulation of orange deposits on the surface of joints and in synovia were considered as a symptom of amyloid arthropathy. The presence of amyloid in tissues was examined using lugol solution technique [21].

Isolation and identification of enterococcus strains: Joint samples and visceral organs of animals dead in observation period and killed by cervical dislocation at the end of this period were collected. Reisolation and identification of enterococcus at species level was performed as described above.

RESULTS

Isolation and Identification

Phenotypic properties of strains were examined for identification of strains isolated from chickens with arthritis and for confirmation of OG1X strains. In Gram staining of brown-black colonies growing on D-Coccocell Agar, Gram positive coccus-shaped cells were occurred in singles or short chains. These micro-organisms were found to be catalase negative both in slide and tube catalase tests. They also have grown at 45°C. The results of carbohydrate fermentation tests in BHI broth showed that strains were mannitol, sorbitol and sucrose positive, sorbose, arabinose and raffinose negative. In motility test, all strains were nonmotile. Telluride tolerance properties of strains were found to be positive in BHI broth containing 0.04% sodium telluride. No yellow pigmentation was occurred on BHI agar. All strains evaluated for their properties mentioned above were identified as *E. faecalis*.

Virulence Factors

Virulence factors of *E. faecalis* strains used in this

Table 1. *The virulence factors of E. faecalis strains used in the study*
Tablo 1. *Çalışmada kullanılan E. faecalis suşlarının sahip oldukları virulens faktörleri*

Strain	Virulence Factor		
	AS	Gelatinase	Cytolisine
E .faecalis OG1RF	-	+	-
E. faecalis OG1X(pAM9058)	+	-	-
E. faecalis OG1SSP	+	+	-
E. faecalis OG1X(pAM714)	+	-	+
E. faecalis OG1X	-	-	-
E. faecalis OG1X(pAM944)	-	-	+
E. faecalis isolate1	-	-	+
E. faecalis isolate2	+	+	+
E. faecalis isolate3	-	-	-
E. faecalis 6085.94	-	+	-

AS: Aggregation substance

study were presented in *Table 1*.

Results of Experimental Infection

The results obtained from intraarticular groups composed of 2 weeks old chickens were presented in *Table 2*. Also, the results obtained from intravenous groups composed of 5 weeks old chickens were presented in *Table 3*.

The means of daily body weight loss of chickens in both intraarticular and intravenous experimental groups were analyzed statistically by Kruskal-Wallis Test and decreasing in body weight gain of groups inoculated with gelatinase positive *E. faecalis* strain was found as significant (P<0.001).

Table 2. *Total demonstration of results by intraarticular inoculation groups*
Tablo 2. *Intraartiküler inokulasyon deney gruplarına göre bulguların toplu sunumu*

Group	Virulence Factor			Arthritis	Amyloidosis		Avarage Body Weight Gain (g)	Reisolation of Bacteria	
	Gel	AS	Cyt		Joint	Viscera		Joint	Viscera
1A	+	-	-	5/5	5/5	0/5	8.54	5/5	0/5
1B	+	-	-	6/6	3/6	0/6	10.41	6/6	0/6
1C	-	+	-	5/5	0/5	0/5	12.38	5/5	0/5
1D	+	+	-	6/6	3/6	0/6	10.30	6/6	0/6
1E	-	+	+	6/6	0/6	0/6	11.91	6/6	0/6
1F	-	-	+	6/6	0/6	0/6	11.83	6/6	0/6
1G	+	+	+	6/6	4/6	0/6	9.76	6/6	0/6
1H	-	-	-	6/6	0/6	0/6	12.06	6/6	0/6
1I	-	-	-	0/6	0/6	0/6	13.35	0/6	0/6

Gel:Gelatinase, Cyt: Cytolisine, AS: Aggregation substance

Table 3. *Total demonstration of results by intravenous inoculation groups.*
Tablo 3. *Intravenöz inokulasyon deney gruplarına göre bulguların toplu sunumu*

Group	Virulence Factor			Arthritis	Amyloidosis		Avarage Body Weight Gain (g)	Reisolation of Bacteria	
	Gel	AS	Cyt		Joint	Viscera		Joint	Viscera
2A	+	-	-	5/5	5/5	5/5	7.52	5/5	5/5
2B	+	-	-	5/5	3/5	3/5	9.34	5/5	5/5
2C	-	+	-	4/4	0/4	0/4	12.15	4/4	4/4
2D	+	+	-	5/5	3/5	3/5	9.28	5/5	5/5
2E	-	+	-	5/5	0/5	0/5	11.54	5/5	5/5
2F	-	+	+	5/5	0/5	0/5	11.46	5/5	5/5
2G	+	+	+	5/5	4/5	4/5	8.86	5/5	5/5
2H	-	-	-	4/4	0/4	0/4	11.60	4/4	4/4
2I	-	-	-	0/5	0/5	0/5	12.68	0/5	0/5

Gel:Gelatinase, Cyt: Cytolisine, AS: Aggregation substance

DISCUSSION

Enterococci were formerly classified as Group D *Streptococcus*. Genomic DNA analysis performed in 1980s indicated that enterococci were differing from streptococci. The separate genus classification was appropriate and *Enterococcus* genus was approved. *E. faecalis* and *E. faecium* are the most common enterococci isolated from the enterococcal infections.

Enterococcus faecalis causes several infections in man and animals. One of the most important enterococcal infections that an *E. faecalis* cause is avian amyloid arthropathy. Amyloid arthropathy is clinically characterized by arthritis. In this study, arthritis was occurred in all chickens inoculated with *E. faecalis* strains varied in their virulence factors. In the other experimental studies, it has also been reported that amyloidosis in joints was characterized with arthritis in 97 percent [22]. In studies on the detection of bacteria responsible for arthritis in rats and rabbits, *E. faecalis* has been found to be associated with arthritis [23,24]. In this study, after the inoculation of *E. faecalis* strains both by intraarticular and intravenous routes, arthritis was occurred in all chickens. Likewise, it has been reported that chickens were infected with *E. faecalis* experimentally by intraarticular [22] and intramuscular [25] routes. All the results show that *E. faecalis* has an affinity for joints of chickens regardless of inoculation routes. Although arthritis was occurred in all chickens inoculated in this study, in the other study, it has been reported that when 10^5 CFU/ml and 10^6 CFU/ml of inoculums were used by intramuscular route, arthritis was occurred in 6.3 and 56.2 percent, respectively [25]. The difference was explained by inoculation with higher dose of inoculums (10^6 CFU/ml for intraarticular route and 10^8 CFU/ml for intravenous route) in this study. In the light of this results arthritis formation doesn't depend on inoculation route, it depends on dose of inoculums. Furthermore, gelatinase, AS and cytolisine positive *E. faecalis* strain caused arthritis in 100 percent and no difference in development of arthritis between strains varied in their virulence factors was observed. These results also show that virulence factors examined in this study have not any role in developing arthritis and all *E. faecalis* strains in adequate dose can cause arthritis.

In this study, in groups inoculated with *E. faecalis* 6085.94 strain as positive control it was found to develop amyloid arthropathy in 100 percent both by two inoculation routes. This strain is the first enterococcus strain which has been isolated for amyloid arthropathy in chickens [14]. In these cases occurred spontaneously, *E. faecalis* has been isolated in 33 percent in infected chickens and it has first been proposed that *E. faecalis* can cause amyloid arthropathy. As the aim of this study was to investigate the effects of virulence factors on the development of amyloid arthropathy, other strains varied in these virulence factors were also used. Strains used in this study caused amyloid arthropathy in 39.4 percent when they were given intravenously, in 32.6 percent when they were given by intraarticular route. In this study, gelatinase was considered as common virulence factor of *E. faecalis* strains which contribute to development of amyloid arthropathy. While the rate of amyloid arthropathy was 65.2% in chickens inoculated with gelatinase positive *E. faecalis* strains by intraarticular route, in groups inoculated with AS positive and cytolisine positive strains, the rates of amyloid arthropathy were 30.2% and 22.2%, respectively. Although the strains used in this study have on or more virulence factors. The cumulative evaluation performed in this study showed clearly that gelatinase was more correlated with the development of amyloid arthropathy. Furthermore no occurrence of amyloid arthropathy in any chickens inoculated with gelatinase negative strains by intra-articular route supported this correlation. While amyloid arthropathy was occurred in 75 percent in chickens inoculated with gelatinase positive strains by intra-venously, the rates of amyloid arthropathy were found as 50% and 26.6% in chickens inoculated with AS positive and cytolisine positive strains, respectively. The correlation between gelatinase and the development of amyloid arthropathy was also determined in intra-venous inoculation groups because of no appearance of amyloid arthropathy in any chickens inoculated with gelatinase negative strains like in intraarticular inoculation groups. The results of this study couldn't compare; because there is no article concerning about the effects of virulence factors on the development of amyloid arthropathy. In one study for determination of the potential of *E. faecalis* strains from different sources in the development of amyloid arthropathy, *E. faecalis* isolated from brain, oviduct and egg yolk have been inoculated to brown layer chickens intra-venously and the differences has been reported in the rates of the development of amyloid arthropathy of *E. faecalis* strains [22]. It has been reported that *E. faecalis* 6085.94 isolate known as arthropathic and amyloidogenic, oviduct isolate and egg yolk isolate caused amyloid arthropathy in 100, 10 and 50 percent,

respectively. However no lesion has been occurred with the inoculation of brain isolate. Although Landman et al.[14,15,22,25,26] have not examined the virulence factors in their study, their results consider that not all *E. faecalis* strains cause amyloid arthropathy and various factors relating to *E. faecalis* strains have effects on the development of amyloid arthropathy. In this study, while intraarticular and intravenous inoculations of AS and cytolisine negative strains to chickens resulted in amyloid arthropathy, in groups inoculated with gelatinase negative strains amyloid arthropathy did not occurred. These results showed that gelatinase has an effect on the development of amyloid arthropathy.

All results showed that *E. faecalis* strains producing gelatinase could cause amyloid arthropathy in chickens characterized by arthritis, lameness and growth retardation. This study concerning about the role of virulence factors in the development of amyloid arthropathy was the first performed worldwide and it was showed that the putting into practice of the results and getting more information about this subject will help for raising of healthy poultry.

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