Characterization of the Pathogenesis Mechanism after *Pseudomonas aeruginosa* Infection through Food Consumption Using Chick Embryo Model

Jinsoo Song, Eun-Jung Jin, and Kyoung-Hee Choi^{1*}

Department of Biological Sciences, College of Natural Sciences, Wonkwang University, Iksan 570-749, Korea

¹Department of Oral Microbiology, College of Dentistry, Wonkwang University, Iksan 570-749, Korea

Abstract

This study introduced a chick embryos' infection model to elucidate the pathogenesis mechanism of *Pseudomonas aeruginosa*, which causes serious diseases in human after ingestion of *P. aeruginosa*-contaminated animal originated foods. The embryonic chick model is able to give a rapid and relatively inexpensive method to assess bacterial pathogenicity compared to embryos of other vertebrates. Embryos were infected with *P. aeruginosa* and elastase-deficient *P. aeruginosa*. After infection with *P. aeruginosa* cells, total bacterial cell numbers and gelatinase activities in the embryos were compared. Thereafter, precartilaginous condensation and chondrogenesis were assessed by peanut agglutinin (PNA) binding on day 3 and by Alcian blue staining for sulfated proteoglycans on day 5, respectively. *P. aeruginosa* significantly increased in embryos, resulting in abnormal limb development, whereas *P. aeruginosa* defective in elastase activity partly impaired proliferation. In addition, *P. aeruginosa*-infected chick embryos significantly stimulated the production of matrix metalloproteinases. Several analyses showed that elevated proteases suppressed the proliferation and survival of chondrogenic cells. The results show that this infection model was a useful assay to determine the virulence mechanism of *P. aeruginosa* in human after intake of microbiologically contaminated foods.

Key words: *Pseudomonas aeruginosa*, chick embryos, elastase, pathogenesis

Introduction

Bacterial infection in humans arises frequently after ingestion of contaminated foods with various pathogens, especially from pork, beef, poultry meat, and eggs (Namata *et al.*, 2009). To escape from the prevalence of bacterial pathogens risking human health, it is necessarily preceded to undertake the problem at the level of live-stock farm, thus diminishing the cross-contamination within a herd or flock (Collard *et al.*, 2007; Namata *et al.*, 2005). Although animals are infected with pathogenic bacteria, they become often asymptomatic, but can be spread readily at the farm (EFSA (European Food Safety Authority), 2007; Namata *et al.*, 2009). In addition to horizontal transmissions of pathogens, bacterial infection can be vertically transmitted from mother to fetus, which is

one of the important routes in the contamination of flocks with pathogenic bacteria (Namata *et al.*, 2009).

According to previous studies, the primary genera found in pasteurized egg products are *Aliccaligenes*, *Bacillus*, *Escherichia*, *Proteus*, *Pseudomonas*, and Gram-positive bacteria (Schmidt-Lorenz, 1983; Cunningham, 1995). Among them, psychrotrophs including the genera *Pseudomonas* primarily cause spoilage of egg whites at refrigerated condition (MacKenzie and Skerman, 1982). Thus, the shelf-life of liquid eggs is generally short at refrigeration temperatures.

Among *Pseudomonas* species, *Pseudomonas aeruginosa* is one of the most significant food spoilage organisms and a ubiquitous, opportunistic human pathogen which is able to cause life-threatening infections in injured, burned, and immunocompromised patients (Myszka and Czaczyk, 2009; Van Delden and Iglewski, 1998). *P. aeruginosa* causes off-flavor in various foods including meats, vegetables, and fish (Bower *et al.*, 1996). Especially, *P. aeruginosa* is known to dominate proteinaceous foods including meat, poultry, milk, and fish stored at chill tem-

*Corresponding author: Kyoung-Hee Choi, Department of Oral Microbiology, College of Dentistry, Wonkwang University, Iksan 570-749, Korea. Tel: 82-63-850-6911, Fax: 82-63-850-7313, E-mail: kheechoi@wku.ac.kr

peratures (Gram *et al.*, 2002). Also, ingestion of *P. aeruginosa*-contaminated foods may result in various diseases such as urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, and bacteremia in humans using various virulence-related factors. Among a number of virulence factors of *P. aeruginosa*, several determinants are required for causing disease in diverse hosts, but others only in specific host species. In addition, since *P. aeruginosa* is an agent of various diseases, evolutionarily divergent host infection models were developed and applied to characterize the factors and virulence mechanism of *P. aeruginosa* relevant to clinical settings.

Among them, genetically accessible invertebrates such as *Caenorhabditis elegans* (roundworm), *Drosophila melanogaster* (fruitfly), and *Dictyostelium discoideum* (amoeba) have been developed to learn *P. aeruginosa* pathogenesis (D'Argenio *et al.*, 2001; Mahajan-Mihlos *et al.*, 1999; Pukatzki *et al.*, 2002). Unlike rodent models, these model hosts possess several advantages which include cost-effectiveness, small size and short life cycle of the organisms, thus enabling tests such as genome-wide genetic screens (Clatworthy *et al.*, 2009). However, the use of the invertebrate hosts is unfeasible because vertebrate immune responses are distinguished from invertebrate ones, such that the latter is not related to adaptive immunity, one of the features of humans (Clatworthy *et al.*, 2009).

A chick embryo, which is vulnerable to infection with many pathogenic bacteria, is one of critical vehicles to conduct mother-to-child transmission of bacterial infection. The embryonic chick model is capable of giving a rapid and relatively inexpensive measure of the toxicity of a number of pathogens. Chick embryos are relatively inexpensive and easy to maintain in a laboratory setting as compared with embryos of other vertebrates. Unlike other higher vertebrate systems, such as mouse ova, fertilized eggs can be sustained in a simple incubator, with need for upkeep or any manipulation of the mother; therefore expenditures of time and money for feeding and cage cleaning are unnecessary. Chicks are also ideal for developmental studies because the embryo is easily accessible and relatively easy to manipulate. Penetration of the egg and access to the developing embryo require much less time and effort than in other vertebrates. In mammals, access to the embryo cannot be acquired without manipulation of the mother, which is generally an invasive procedure that is stressful and potentially dangerous for both the mother and the developing embryo. Thus, the chicken embryo provides an excellent model for the study of the virulence of pathogens for humans, such as *Neisseria*

gonorrhoeae and *Neisseria meningitidis* (Buddingh, 1970; Bumgarner and Finkelstein, 1973; Diena *et al.*, 1975; Fransch *et al.*, 1976). Chick embryos have been used to test host-related bacterial virulence and pathology in a wide variety of bacteria such as *Francisella* spp., *Mycoplasma lipofaciens*, *N. gonorrhoeae*, *N. meningitidis*, *Streptococcus flexneri*, *Escherichia coli* and *Vibrio cholerae* (Lierz and Hafez, 2008; Nix *et al.*, 2006; Payne and Finkelstein, 1978). The use of embryonated hens' eggs was performed also in *P. aeruginosa* infection to screen antibacterial therapeutic substances as an alternative *in vivo* model (Hartl *et al.*, 1997). Up to date, however, it has not been utilized to define important molecular mechanisms regarding virulence of *P. aeruginosa* in eggs.

Therefore, our objective in this work was to develop a useful assay system that tests the virulence and pathogenesis mechanism of *P. aeruginosa*, and elucidates specific features playing a major role in a human-pathogen interaction. This further will suggest an effective way to control food spoilage and vertical transmission of bacterial infection, ultimately contributing to human health.

Materials and Methods

Bacterial strains and growth conditions

Escherichia coli DH5 α and *P. aeruginosa* PA14 strains (including wild-type, *lasB* and *gfp* mutants) were maintained on Luria-Bertani medium (LB; 10 g per liter tryptone, 5 g per liter yeast extract, 10 g per liter NaCl; Difco) at 37°C incubator. *P. aeruginosa* PA14 strain containing a chromosomal copy of green fluorescence protein (*gfp*) was created using mini-Tn7 gene integration system as previously described (Choi and Schweizer, 2006).

Bacterial inoculation into chicken embryos

Specific pathogen-free fertile eggs from White Leghorn chickens fed antibiotic-free meals were incubated in a Humidaire incubator (Saesil, Gyeonggi-do, Korea) with automatic turning, controlled temperature (37.3 to 37.8 °C), humidity (50 to 55%), and air circulation. The age of the embryos was determined morphologically by using the Hamburger series of normal stages (Hamburger and Hamilton, 1951) in the chicken embryo, with a minimum of three eggs per experiment sacrificed for this purpose.

Nine day-old chick embryos were selected for inoculation. The eggs were opened by making a window over the chorioallantoic membrane and allowing the membrane to recede. They were inoculated with a sublethal infection dose, 10⁴ CFU of *P. aeruginosa* cells, and the window

was covered with adhesive tape. At 24 h after inoculation, suspensions of embryos, 20% by weight, were made by grinding the embryo in a Waring blender (Waring blender, Torrington, CT, USA) in cold saline buffered with phosphate. Such suspensions were centrifuged in the Swedish angle centrifuge at about 3,000 g. for 15 min before titration of the supernatant.

Bacterial enumeration from infected embryos

Dead embryos were removed and survived individual chick embryos were collected. The eggs were blended followed by plating dilutions of the homogenized egg extracts on LB plates to enumerate colony forming units (CFU) within infected eggs. Total bacterial numbers were standardized by dividing by each egg weight.

Gelatin zymography

Gelatinase activity was determined using the gelatin zymography assay with 0.1% gelatin (Sigma, St. Louis, Mo, USA) as a substrate in 10% SDS-polyacrylamide gel. Embryo lysates was subjected to SDS-PAGE under non-reducing conditions. After electrophoresis, gels were washed three times with 2.5% Triton X-100 (Sigma) in water, then incubated with 50 mM Tris buffer, pH 7.4, containing 0.2% Brij 35, 5 mM CaCl_2 , and 1 mM NaCl, overnight at 37°C in a closed container. Gels were stained for 30 min with 0.25% Coomassie Blue R-250 (Amresco, Cochran Road Solon, OH, USA) in 10% acetic acid and 45% methanol, and destained for 30 min using a mixture of 20% acetic acid, 20% methanol and 17% ethanol. Areas of gelatinase activity were detected as clear bands against the blue-stained gelatin background.

Cell culture and cell proliferation assay

For limb mesenchymal cell culture, the modified technique of Ahrens *et al.* (1993) was used. Briefly, micro-mass cultures used 10 μL drops of cells at 10^7 cells/mL maintained in Ham's F-12 medium (Gibco Invitrogen, Grand Island, NY, USA) containing 10% FBS. Micro-mass cultures were also incubated with or without purified gelatinase induced by inoculation. Gelatinase concentration was achieved by ultrafiltering the sample solution through Ultracel YM-30 membrane (Millipore, Concord Road, Billerica, MA, USA).

Proliferation of mesenchymal cells was determined by direct counting of cells from micromass cultures. Control and treated cultures were maintained for the indicated number of days, detached with trypsin/EDTA (Gibco, Invitrogen) solution and counted in triplicate using a hema-

cytometer (Superior, Lauda-Königshofen, Germany).

Analysis of cell differentiation

Chondrogenesis was measured by Alcian blue (Sigma) staining of sulfated cartilage glycosaminoglycans. Alcian blue-bound sulfated glycosaminoglycans were extracted with 6 M guanidine-HCl (Sigma), and quantified by measuring the absorbance of the extracts at 600 nm by spectrophotometer (Tecan, Männedorf, Switzerland).

Results

Morphological defect during developmental progression of chicken embryos after infection with *P. aeruginosa*

Nine day-old chick embryos were infected with various doses of wild-type *P. aeruginosa* and *E. coli* strain (EC) ranging from 10^2 to 10^7 CFU at an interval of a logarithmic dilution. As a result, embryonated eggs were more fatal to *P. aeruginosa* than to *E. coli*, and unlike *E. coli*, 10^4 CFU of *P. aeruginosa* cells was shown as a sublethal dose in embryonated eggs (data not shown). At the infection dose, chick embryos were survived at 24 h after infection, while they became dead over 24 h incubation (data not shown). Therefore, chick embryos were incubated with an inoculation of 10^4 CFU of bacterial cells for 24 h in order to observe a severe and clear effect of bacterial pathogenesis including morphological defect during developmental progression of embryonated eggs. Wild-type *P. aeruginosa* strain survived and proliferated four-fold more than nonpathogenic *E. coli* strain within chick embryos (Fig. 1A). The bacterial infectivity was visualized with paraffin sectioning and gram staining of chicken head and body infected with *P. aeruginosa* cells (Fig. 1B). *P. aeruginosa* cells successfully established their growth within both chicken head and body.

Elastase, encoded by *lasB* gene, is one the most significant invasive factor during *P. aeruginosa* infection (Van Delden, 2004). Therefore, the virulence of wild-type and its *lasB* mutant were compared. Infection rates of *P. aeruginosa* and *lasB* mutant in chicken embryos were compared by recovering the embryos and enumerating bacterial cells (Fig. 1A). As a result, although wild-type and its *lasB* mutant grow at a same rate (data not shown), the *lasB* mutant displayed two-fold decreased infection rate compared to the wild-type *P. aeruginosa* strain. However, the infectivity of the *lasB* mutant was still relatively high compared to one of the *E. coli* strain. It is thought to be due to a relatively low level of elastolytic activity by

Fig. 1. A. Bacterial enumeration from infected embryos. Chick embryos were infected with *E. coli* DH5a (EC), *P. aeruginosa* (PA), *P. aeruginosa* defected in elastase activity (*lasB*). Ratio of CFU/mL shown on the Y-axis is calculated by dividing each CFU/mL by the one of EC. **B. Paraffin section and gram staining of chicken head and body infected with *P. aeruginosa*.** Area of *P. aeruginosa* cells was shown in pink colors.

LasA (Kessler and Safrin, 1997).

The malformation of limb also was observed when HH stage 31 chick embryos were inoculated with *P. aeruginosa* for 24 h, suggesting the defection on the process of endochondral ossification by infection of invasive *P. aeruginosa* (Fig. 2).

***P. aeruginosa*–induced gelatinase stimulates apoptotic death of chondrogenic progenitors**

To determine whether inoculation of chick embryos with *P. aeruginosa* lead to induction of gelatinase, zymography was performed (Fig. 3A). Lysates of *P. aeruginosa*-inoculated chick embryo showed distinct bands on gelatin gels, indicating the presence of several Matrix metalloproteinase (MMP)s with gelatinolytic and/or collagenolytic activity of 30-50 kDa proteins compared to EC-inoculated embryo. This indicated that this increased activity might be responsible for virulent actions of *P. aeruginosa* including abnormal limb development. According to previous studies, corneas were destructed with infection of *P. aeruginosa* by the mechanisms that *P. aeruginosa* exproteases stimulated expression of MMPs releas-

Fig. 2. Morphological defect during developmental progression of chicken embryos after infection with *P. aeruginosa*.

ed by corneal stromal cells and *P. aeruginosa* elastase proteolytically activated MMPs (Miyajima *et al.*, 2001).

To confirm this, proteases activated by inoculation of *P. aeruginosa* were purified and used for determining the effects on chondrogenesis. Chondroblasts isolated from wing buds were cultured at a density of 2×10^7 cells/mL and stimulated with purified proteases. Precartilag condensation and chondrogenesis were assessed by peanut agglutinin (PNA) binding on day 3 and by Alcian blue staining for sulfated proteoglycans on day 5, respectively. With treated of *P. aeruginosa*-induced protease, PNA staining and Alcian blue uptake were significantly decreased (Fig. 3B). These results demonstrate that *P. aeruginosa*-induced protease inhibits sulfated proteoglycan accumulation and cartilage nodule formation during chondrogenesis.

Precartilag condensation is known to be regulated by cell-density due to cell proliferation or apoptotic cell death (Maini and Solursh, 1991; Solursh, 1989). We first examined if *P. aeruginosa*-induced protease-inhibited chondrogenesis was due to regulation of cell proliferation. During chondrogenic differentiation, the cell numbers increased in control cultures. Exposure of cells to *P. aeruginosa*-induced protease suppressed the proliferation of chondrogenic competent cells as determined by direct cell counting (Fig. 3C). These data indicate that the negative

Fig. 3. A. Gelatin zymography. Embryo lysates after infection with *E. coli* DH5 α (EC), *P. aeruginosa* (PA), *P. aeruginosa* defected in elastase activity (*lasB*) were used for the gelatin zymography. **B. Effect of *P. aeruginosa*-induced protease on sulfated proteoglycan accumulation and cartilage nodule formation during chondrogenesis.** After exposure of cells to *P. aeruginosa*-induced protease, precartilage condensation and chondrogenesis were assessed by peanut agglutinin (PNA) binding and by Alcian blue staining for sulfated proteoglycans. **C. Effect of suppressed chondrogenesis induced by the protease on the proliferation and survival of chondrogenic competent cells by direct cell counting.**

regulatory action by *P. aeruginosa*-induced protease results by modulating the proliferation and survival of chondrogenic competent cells.

Discussion

In this study, we show that chick embryos can be utilized as an infection host for elucidating the mechanism

of bacteria-mediated pathogenesis. So far, its use has been limited to test bacterial pathogenicity and to screen antibacterial drugs in various bacteria (Lierz and Haffez, 2008; Nix *et al.*, 2006; Payne and Finkelstein, 1978; Hartl *et al.*, 1997). However, a usefulness of its application successfully expanded into the characterization of central molecular mechanisms regarding bacterial virulence in the present study.

P. aeruginosa cells successfully survived and proliferated in chicken head and body for 24 h after inoculation of bacterial cells into chick embryos. The infectivity of the *lasB* mutant was relatively low compared to one of the wild-type *P. aeruginosa* strain. During *P. aeruginosa* infection, proteases play a major role in virulence and tissue penetration (Tang *et al.*, 1996; Twinning *et al.*, 1993). *P. aeruginosa* synthesizes several proteases, including LasA and LasB elastases, and alkaline protease. Elastin present in all invertebrates is a principal protein of connective tissues and its degradation is carried out by the LasB enzyme, which is known to be an important enzyme with elastolytic activity (Van Delden, 2004; Kessler and Safrin, 1997). Particularly, LasB elastase degrades not only elastin but also collagen, which is another major component of connective tissues (Heck *et al.*, 1986). Elastases were determined as a main virulence determinant of causing the pulmonary hemorrhages during invasive *P. aeruginosa* clinical settings (Van Delden, 2004). Cowell *et al.* (2003) showed that mutation of *lasB* gene encoding LasB enzyme suppressed *P. aeruginosa* invasion. In addition, previous studies demonstrated that *P. aeruginosa lasB* mutant exhibited decreased virulence in animal models (Tang *et al.*, 1996; Tamura *et al.*, 1992).

In addition, chick embryos suffered the morphological defect on limb during developmental progression of chicken embryos after infection with *P. aeruginosa*. It is suggested that the malformation of chicken limb resulted from the invasiveness of the bacteria by elastolytic action on embryonic connective tissues. However, how it mediates the morphological defect during limb formation is not clear at this time. At the molecular level, *P. aeruginosa* cells significantly stimulated the production of several matrix metalloproteinases, indicating the possible role in bacterial virulence such as deformed limb development. Considerably increased matrix metalloproteases played a negative role in sulfated proteoglycan accumulation and cartilage nodule formation during chondrogenesis through the down-regulation of chondrogenic cell proliferation. However, a better and complete understanding of the bacteria-mediated pathogenic mechanism should

be accomplished in future work.

Taken together, chick embryo infection model was very useful to establish the bacterial virulence mechanism by examining the morphological effect and the molecular change via host-bacteria interaction. In addition, the study will provide an idea to prevent food spoilage and life-threatening disease in human by controlling bacterial infection and spread.

Acknowledgement

This study was supported by a grant of the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A084717).

References

- Ahrens, M., Ankenbauer, T., Schröder, D., Hollnagel, A., Mayer, H., and Gross, G. (1993) Expression of human bone morphogenetic proteins-2 or -4 in murine mesenchymal progenitor C3H10T1/2 cells induces differentiation into distinct mesenchymal cell lineages. *DNA Cell. Biol.* **12**, 871-880.
- Bower, C. K., McGuire, J., and Daeschel, M. A. (1996) The adhesion and detachment of bacteria and spores on food-contact surfaces. *Trends Food Sci. Tech.* **7**, 152-157.
- Buddingh, G. J. (1970) The chick embryo for the study of infection and immunity. *J. Infect. Dis.* **121**, 660-663.
- Bumgarner, L. R. and Finkelstein, R. A. (1973) Pathogenesis and immunology of experimental gonococcal infection: virulence of colony types of *Neisseria gonorrhoeae* for chicken embryos. *Infect. Immun.* **8**, 919-924.
- Choi, K. H. and Schweizer, H. P. (2006) mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa*. *Nat. Prot.* **1**, 153-161.
- Clatworthy, A. E., Lee, J. S., Leibman, M., Kostun, Z., Davidson, A. J., and Hung, D. T. (2009) *Pseudomonas aeruginosa* infection of zebrafish involves both host and pathogen determinants. *Infect. Immun.* **77**, 1293-1303.
- Collard, J. M., Bertrand, S., Dierick, K., Godard, C., Wilde-mauwe, C., Vermeersch, K., Duculot, J., Van Immerseel, F., Pasmans, F., Imberechts, H., and Quinet, C. (2007) Drastic decrease of *Salmonella Enteritidis* isolated from humans in Belgium in 2005, shift in phage types and influence on food-borne outbreaks. *Epidemiol. Infect.* **136**, 771-781.
- Cowell, B. A., Twining, S. S., Jeffrey, A. H., Kwong, M. S. F., and Fleiszig, S. M. J. (2003) Mutation of *lasA* and *lasB* reduces *Pseudomonas aeruginosa* invasion of epithelial cells. *Microbiol.* **149**, 2291-2299.
- Cunningham, F. E. (1995) Egg-product pasteurization. In: *Egg science and technology*. Stadelman, W. J. and Cotterill, O. J. (eds) Food Products Press, NY, 289-322.
- D'Argenio, D. A., Gallagher, L. A., Berg, C. A., and Manoli, C. (2001) *Drosophila* as a model host for *Pseudomonas aeruginosa* infection. *J. Bacteriol.* **183**, 1466-1471.
- Diena, B. B., Lavergne, G., Ryan, A., Ashton, F. E., Wallace, R., and Perry, M. (1975) The chick embryo in studies of virulence and immunity with *Neisseria gonorrhoeae*. *Rev. Can. Biol.* **34**, 213-220.
- EFSA (European Food Safety Authority) (2007) *Salmonella*. The community summary report on trends and sources of zoonoses, zoonotic agents, antimicrobial resistance and foodborne outbreaks in the European Union in 2005. 27-81.
- Frasch, C. E., Parkes, L., McNelis, R. M., and Gotschlick, E. C. (1976) Protection against group B meningococcal disease. I. Comparison of group-specific and type-specific protection in the chick embryo model. *J. Exp. Med.* **144**, 319-329.
- Gram, L., Ravn, L., Rasch, M., Bruhn, J. B., Christensen, A. B., and Givskov, M. (2002) Food spoilage—interactions between food spoilage bacteria. *Int. J. Food Microbiol.* **78**, 79-97.
- Hamburger, V. and Hamilton, H. L. (1951) A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.
- Hartl, A., Mollmann, U., Schrinner, E., and Stelzner, A. (1997) *Pseudomonas aeruginosa* in embryonated hen's eggs. An alternatives *in vivo* model for the screening of antibacterial substances. *Arzneimittelforschung* **47**, 1061-1064.
- Heck, L. W., Morihara, K., McRae, W. B., and Miller, E. J. (1986) Specific cleavage of human type III and IV collagens by *Pseudomonas aeruginosa* elastase. *Infect. Immun.* **51**, 115-118.
- Kessler, E. and Safrin, M. (1997) Inhibitors and specificity of *Pseudomonas aeruginosa* LasA. *J. Biol. Chem.* **272**, 9884-9889.
- Lierz, M., and Hafez, H. M. (2008) Time-dependent recovery of *Mycoplasma lipofaciens* (strain ML64) from incubated infertile chicken eggs and dead in shell chicken embryos. *Avian Dis.* **52**, 441-443.
- Mackenzie, K. A. and Skerman, V. B. D. (1982) Microbial spoilage in unpasteurised liquid whole egg. *Food Technol. Aust.* **34**, 524-528.
- Mahajan-Miklos, S., Tan, M. W., Rahme, L. G., and Ausubel, F. M. (1999) Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa*-*Caenorhabditis elegans* pathogenesis model. *Cell* **96**, 47-56.
- Maini, P. K. and Solursh, M. (1991) Cellular mechanisms of pattern formation in the developing limb. *Int. Rev. Cytol.* **129**, 91-133.
- Miyajima, S., Akaike, T., Matsumoto, K., Okamoto, T., Yoshitake, J., Hayashida K., Negi, A., and Maeda, H. (2001) Matrix metalloproteinases induction by pseudomonal virulence factors and inflammatory cytokines *in vitro*. *Microb. Pathog.* **31**, 271-281.
- Namata, H., Welby, S., Aerts, M., Faes, C., Abrahantes, J. C., Imberechts, H., Vermeersch, K., Hooyberghs, J., Méroc, E., and Mintiens, K. (2009) Identification of risk factors for the prevalence and persistence of *Salmonella* in Belgian broiler chicken flocks. *Prev. Vet. Med.* **90**, 211-222.
- Nix, E. B., Cheung, K. K. M., Wang, D., Zhang, N., Burke, R. D., and Nano, F. E. (2006) Virulence of *Francisella* spp. Chicken embryos. *Infect. Immun.* **74**, 4809-4816.

26. Payne, S. M., and Finkelstein, R. A. (1978) The critical role of iron in host-bacterial interactions. *J. Clin. Invest.* **61**, 1428-1440.
27. Pukatzki, S., Kessin, R. H., and Mekalanos, J. J. (2002) The human pathogen *Pseudomonas aeruginosa* utilizes conserved virulence pathways to infect the social amoeba *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. USA* **99**, 3159-3164.
28. Schmidt-Lorenz, W. (1983) Collection of methods for the microbiological examination of foods. Verlag Chemie, Weinheim, Germany, 15.1-15.22
29. Solursh, M. (1989) Differentiation of cartilage and bone. *Curr. Opin. Cell. Biol.* **1**, 989-994.
30. Tamura, Y., Suzuki, S., and Sawada, T. (1992) Role of elastase as a virulence factor in experimental *Pseudomonas aeruginosa* infection in mice. *Microb. Pathog.* **12**, 237-244.
31. Tang, H. B., Dimango, E., Brian, M. J., Gambello, M. J., Iglewski, B. J., Goldberg JB, and Prince, A. (1996) Contribution of specific *Pseudomonas aeruginosa* virulence factors to pathogenesis of pneumonia in a neonatal mouse model of infection. *Infect. Immun.* **64**, 37-43.
32. Twinning, S. S., Kirschner, S. E., Mahnke, L. A., and Franke, D. W. (1993) Effect of *Pseudomonas aeruginosa* elastase, alkaline protease, and exotoxin A on corneal proteinases and proteins. *Invest. Ophthalmol. Vis. Sci.* **34**, 2699-2712.
33. Van Delden, C. V. (2004) Virulence factors in *Pseudomonas aeruginosa*. In: *Pseudomonas: virulence and gene regulation*. Kluwer Academic/Plenum Publishers, New York, pp. 23-45.
34. Van Delden, C., and Iglewski, B. H. (1998) Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerg. Infect. Dis.* **4**, 551-560.

(Received 2010.4.22/Revised 2010.5.27/Accepted 2010.5.28)