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

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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, precartilage 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 livestock 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 *Alicaligenes*, *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 temperature.
peratures (Gram et al., 2002). Also, ingestion of \textit{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 \textit{P. aeruginosa}, several determinants are required for causing disease in diverse hosts, but others only in specific host species. In addition, since \textit{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 \textit{P. aeruginosa} relevant to clinical settings.

Among them, genetically accessible invertebrates such as \textit{Caenorhabditis elegans} (roundworm), \textit{Drosophila melanogaster} (fruitfly), and \textit{Dictyostelium discoideum} (amoeba) have been developed to learn \textit{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 \textit{Neisseria gonorrhoeae} and \textit{Neisseria meningitidis} (Buddingh, 1970; Bumgarner and Finkelstein, 1973; Diena et al., 1975; Frisch et al., 1976). Chick embryos have been used to test host-related bacterial virulence and pathology in a wide variety of bacteria such as \textit{Francisella} spp., \textit{Mycoplasma lipoferiens}, \textit{N. gonorrhoeae}, \textit{N. meningitidis}, \textit{Streptococcus flexneri}, \textit{Escherichia coli} and \textit{Vibrio cholerae} (Lierz and Hafez, 2008; Nix et al., 2006; Payne and Finkelstein, 1978). The use of embryonated hens’ eggs was performed also in \textit{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 \textit{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 \textit{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.

\section*{Materials and Methods}

\subsection*{Bacterial strains and growth conditions}

\textit{Escherichia coli} DH5α and \textit{P. aeruginosa} PA14 strains (including wild-type, \textit{lasB} and \textit{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. \textit{P. aeruginosa} PA14 strain containing a chromosomal copy of green fluorescence protein (\textit{gfp}) was created using mini-Tn7 gene integration system as previously described (Choi and Schweizer, 2006).

\subsection*{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, 10^4 CFU of \textit{P. aeruginosa} cells, and the window