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Real Time Quantitative Detection for Enterococcus in the Gastrointestinal Tract of Pigeon after Orally Infected by *Salmonella enteritidis*

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Abstract: This research was undertaken to understand the effect of *Salmonella enteritidis* (SE) on the Enterococcus counts in the gastrointestinal tract of China racing pigeon. Researchers applied a FQ-PCR assay to detect the genomic DNA of Enterococcus which extracted from duodenum, jejunum, ileum, cecum and rectum of 3 weeks old pigeon after orally injection by SE at different time points. The results showed that Enterococcus were consistently detected from all segments of the gastrointestinal tract. It mainly distributed in the cecum of the gastrointestinal tract of pegion. At 12-72 h postinfection there were obviously undulation change of the Enterococcus counts. The Enterococcus populations in the jejunum were decreased at 24 h postinfection ($p<0.05$). At 6 days postinfection, the residual Enterococcus can re-grow to normal condition. The research indicated that the Enterococcus counts in the gastrointestinal tract were decreased after SE-infected. The residual Enterococcus can re-grow, outcompete SE and thereby re-establish colonization resistance.

Key words: *Salmonella enteritidis*, gastrointestinal tract, Enterococcus, pigeon, PCR, China

INTRODUCTION

Natural infection of poultry by *Salmonella enterica* serovar *enteritidis* (SE) occurs via oral route and the key step is to disturb the gastrointestinal microbial community of the host, attach and penetrate to the intestinal mucosa and invades the lamina propria (Bohnhoff *et al*., 1964). The gastrointestinal microbial community plays an important role in the growth and health of the birds (Marcinakova *et al*., 2004). A high density of commensal microbiota inhabits the intestine and shields from infection by certain means such as colonization resistance, adjustment to the pH of intestinal lumen, facilitating intestines peristalsis and immunity (Klaasen *et al*., 1993). However, the virulence strategies allowing enteropathogenic bacteria to successfully compete with the microbiota and overcome colonization resistance. For instance, Salmonella can weaken the adhesive capability of lactobacillus to intestinal epithelium (Vesterlund *et al*., 2005). Generally, little is known about the composition and dynamics of the intestinal microbial community of pigeon after SE-infected.

Enterococcus belong to Gram-positive bacterium which keeps the ratio of aerobian and anaerobic bacteria by creating anaerobic living condition to Bifidobacterium and lactobacillus. Enterococcus are considered as key commensal bacterials that promote a healthy gastrointestinal tract (Devriese *et al*., 1991). Studies on the relationship of SE and Enterococcus have traditionally been carried out using co-culture technology *in vitro*, they demonstrated that Enterococcus can inhibit multiplication of SE. Thus, Enterococcus can be used as probiotics to prevent SE. However, the development of Enterococcus in the host's gastrointestinal tract by SE infected is still unknown. The aim of the present study was to examine and identify differences in the development of the Enterococcus of the gastrointestinal tract of pigeon artificially infected with SE compared with uninfected control pigeon.

MATERIALS AND METHODS

Bacterial strains: SE (No. DD126) and Enterococcus (No. CICC 6072) were purchased from China Center of Industrial Culture Collection.

Primer and TaqMan probe design: Based on the previous study, there was designed the Primers and TaqMan probe directly at Enterococcus *23SrRNA* gene (Zhang, 2006). All primers and TaqMan probe were synthesized by gene core bio technologies Co Ltd. (Shanghai, China). Polymerase chain reaction amplifications were performed

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using primer pair (FP: 5'-TTGCATGCGAGGTTAAGTTGA-3',RP: 5'-GAATGGCATTCGGAGTTTATCTG-3'),resulting in fragments of 337 bp; FQ-PCR were performed using primer pair (FP: 5'-ATTCCAAACGAACTGGGAGAT-3', RP: 5'-CCAAACAGTGCTCTACCTCCA-3'), resulting in fragments of 94 bp; probe sequence: (5'-TGGTTCTCTCC GAAATA GCTTTAGGGCTA-3'), the dye-target combinations are as follows: 5'-Carboxyfluorescein (FAM), 3'-Tetramethycarbo xyrhodamine (TAMRA).

TaqMan PCR conditions: TaqMan PCR was performed in a 25 μL reaction volume containing 5×PCR buffer 5 μL, 250 mmol L^{-1} $MgCl_2$ 0.5 μL, 10 mmol L^{-1} dNTPs 0.5 μL, 0.25 μL each primer (20 μmol L^{-1}), 10 μmol L^{-1} TaqMan probe 0.5 μL, 1 U Taq DNA Polymerase (TaKaRa Taq™, TakaRa Biotech Co., Ltd, DaLian, China) and 1 μL templates. A standard cycling protocol (95°C 3 min, 94°C 30 sec, 60°C for 30 sec for maximum of 40 cycles) was used for amplification on a Bio-Rad iQ.

FQ-PCR standard curve: Based on the previous study (Zhang, 2006). The 337 bp amplification product was purified by conventional PCR. Ten fold serial dilutions ranging from 6×10^8-6×10^3 gene copies were as standards template. By using standards template, accurate results for a series of samples were obtained based on the datas to generate the standard curve through the software of iCycler IQ Detection system. The correlation coefficient for the associated standard curve was 0.977 and PCR efficiency was 97.3% indicating that the crossing threshold values for the standards fell within accurate. Through the formula as followed there can quantitate unknown samples, Y = -3.417X+41.565 (where Y is the threshold cycle and X is the log of the starting quantity).

Experimental infection and sampling: Prior to challenge with SE, all pigeons (China racing pigeon) were found to be negative for SE-specific antibodies and SE-specific antigens by an enzyme-linked immunosorbent assay and polymerase chain reaction, respectively (Deng *et al*., 2008; Gast and Beard, 1990). The pigeons were maintained in isolation units in a biosecure animal building.

The 96 pigeons (3 weeks old) were subdivided into two groups. One groups of 48 pigeons was infected orally with a SE strain (No. DD126) at 4×10^8 cell per pigeon. Another group of 48 pigeons was treated with an equal volume of 0.9% NaCl solution as a control. At 30 min, 1, 2, 4, 8, 12, 24, 36, 48, 72 h and 6 and 9 days there collected duodenum, jejunum, ileum, cecum and rectum samples (1 cm) then splited the enteric cavity and exposed the intestinal contents sufficiently. The intestinal content was suspended in 10 mL of PBS. The suspension was mixed thoroughly and centrifuged at 650 r min^{-1} for 10 min. The supernatant was transferred to a new Eppendorf tube and stored at -20°C until further processing.

DNA extraction: Before extracted with phenol-chloroform, there were removed the inhibitory factors for PCR by liquor pyroaceticus. The supernatant was centrifuged at 10000 r min^{-1} for 5 min then collected the pellet and lysed in 2 mL PBS. After 10000 r min^{-1} for 5 min, the pellet was transferred to a new tube containing 100 μL of TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0) and 40 μL lysozyme (10 mg mL^{-1}) and placed in a water bath for 30 min at 37°C. Then 500 μL lysate (200 mmol L^{-1} NaCl) 100 mmol L^{-1} Tris-HCl, pH 8.0, 2.0% SDS, 50 mmol L^{-1} EDTA, 1.0% Triton X-100) and 12.5 μL protease K (20 mg mL^{-1}) was added. The suspension was mixed thoroughly and placed in a water bath for 16 h at 37°C. The sample was extracted 3 times with 0.3 mL of chloroform:isoamyl alcohol (24:1), 0.3 mL of phenol: chloroform:isoamyl alcohol (25:24:1) and 0.6 mL of chloroform:isoamyl alcohol (24:1), respectively (Sigma-Aldrich). The DNA was precipitated with 99.9% cold ethanol. The DNA was dissolved in 50 μL of TE-buffer and stored at -20°C until further use. Finally, a 1 μL aliquor of the DNA extract was used as a template in the FQ-PCR assay as describe earlier.

Statistical analysis: The polymerase chain reaction assay and data acquisition and analysis were performed using the iCycler iQ Optical system software (version 3.1; Bio-Rad, USA). The number of target copies in the reaction was deduced from the threshold cycle values. The threshold cycle value corresponds to the fractional cycle number at which the fluorescence emission exceeds the standard deviation of the mean baseline emission by 15 fold. All samples were analyzed 4 times by the real-time polymerase chain reaction assay and concentrations of the target deoxyribonucleic acid detected were expressed as the mean $\log_{10}$ of the Enterococcus genome copy number per centimeter of intestinal section tested. The real-time polymerase chain reaction data were analyzed using version 11 of the SPSS software. The comparison of means was performed using Duncan's multiple-range test. A $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Clinical signs and gross lesions at necropsy: SE inoculated pigeons appeared to be clinically normal and there were no signs of depression or diarrhea moreover, the feeding and drinking behaviors were normal at 30 min until 12 h and at 3 day until 9 days PI. However, at 12 h and until 2 days PI there were clinical signs of SE

infection. At necropsy, gross lesions were observed in all of the pigeon during this period e.g., intestinal hyperemia and swelling of the gallbladder.

The development of Enterococcus after SE-infected: The count of Enterococcus within the gastrointestinal tract after oral challenge was determined by means of FQ-PCR over a 9 days period at intervals. The results showed that Enterococcus were consistently detected from all segments of the gastrointestinal tract. It mainly distributed in the cecum of the gastrointestinal tract of pegion. At 12-72 h postinfection there were obviously undulation change of the Enterococcus counts. The Enterococcus populations in the jejunum were decreased at 24 h postinfection ($p<0.05$). At 6 day postinfection, the residual Enterococcus can re-grow to normal condition.

For the duodenum, the variance of the Enterococcus counts increased at 12 h postinfection then decreased at 48 h postinfection. There were on dominating variance of the Enterococcus counts from the jejunal contents in the infection group. For the ileum, the Enterococcus counts increased at 12 h postinfection then decreased at 24 h postinfection. The dominating variance of the Enterococcus counts from the rectum contents was increase. At 24 h postinfection there were growth in the orally infection group ($p<0.05$). The Enterococcus counts in orally infection group were lower than the control group, the varietal-regularity was consistent with the control group. However, the Enterococcus counts in cecum were higher than other intestinal section. The details are shown in Table 1.

In this study, Enterococcus were consistently detected from all segments of the gastrointestinal tract. However, it mainly distributed in the cecum of the gastrointestinal tract of pigeons. This may be explained by difference in the anatomic structure of the gastrointestinal tract (He *et al*., 2011a, b). The cecum is a blind sac with a long retention time of the feed whereas the jejunum and ileum is a straight intestinal canal with a much higher flow rate. Thus, the environment of the cecum is more stable than the environment of the others. Throughout the course of the experiment, the Enterococcus counts maintained steady state in the gastrointestinal tract. The Enterococcus counts in ileum increased gradually along with the increase of the pigeon's age. The Enterococcus counts in cecum reached peak amplitude in 9 days postinfection then decreased and maintained steady state.

In the present study, the Enterococcus counts in 5 segments of the gastrointestinal tract of pigeon was investigated following an artificially infection with SE using FQ-PCR. It showed that the level of colonization with Enterococcus decreased in the period of onset (12-72 h postinfection), compared with the negative pigeons. It was probably that the adhesive capability of the Enterococcus to the epithelial tissues was decreased. The same result as Miroslava (He and Wang, 2000; Marcinakova *et al*., 2004) detected in Quail after salmonella-infected. *Inv* gene of SE mediated it attach to and invade the host enterocyte then destroy the structure of the cell. The enterotoxin of SE triggered secretory reaction of the enterocyte cell, resulted to fluidify in enteric cavity. The reactions altered conditons in the intestinal lumen. Released antibacterial factors may kill or retard growth of the normal gut microbiota that would normally inhibit SE growth in the healthy intestine. However, numerous SE genes that function to enhance antimicrobial peptide resistance and radical detoxification (Navarre *et al*., 2005).

In healthy intestine, the normal microflora is shaped and stabilized by mutually beneficial interactions with the intestinal mucosa. The incoming pathogen and the resident microbiota compete for growth. Stecher *et al*. (2007) and Johansen *et al*. (2006) indicated that in order to guarantee its growth, SE suppressed the growth of microbita. The growth competition may be involved into the poor adhesive capability of the Enterococcus. However, loss of the Enterococcus that might be disrupted the whole commensal network of gastrointestinal tract. The present researchs demonstrated

Table 1: Log_{10} 23S rDNA copies of Enterococcus in the intestinal tract of pigeon

	Control group					Orally infection				
sampling time	Duodenum	Jejunum	Ileum	Cecum	Rectum	Duodenum	Jejunum	Ileum	Cecum	Rectum
30 min	5.92	6.60	6.86	7.93	6.15	6.02	6.410	6.58	8.00	6.09
1 h	5.86	6.41	6.80	8.01	6.21	5.15	5.570	6.19	7.31	5.31
2 h	5.88	6.50	7.12	8.50	6.44	5.64	6.250	6.43	7.16	5.46
4 h	5.13	5.81	6.40	6.75	5.22	5.42	5.250	5.45	5.45	5.60
8 h	5.67	5.94	6.13	6.31	5.80	5.32	5.270	5.41	5.44	5.60
12 h	5.56	6.37	6.40	6.59	6.04	6.90	6.380	7.47	6.50	6.30
24 h	5.65	5.88	5.91	6.04	5.54	6.01	5.390	5.40	6.23	6.76
36 h	5.73	5.73	6.04	6.19	5.60	6.61	6.600	6.42	6.01	7.43
48 h	5.70	5.76	5.90	6.21	5.60	5.04	5.190	5.18	5.14	5.57
72 h	5.15	5.53	5.75	6.90	5.17	5.35	5.600	5.76	6.10	5.54
6 days	6.37	6.40	6.36	7.13	5.54	5.13	5.490	5.94	6.17	5.57
9 days	6.10	6.50	7.01	7.90	5.63	6.16	6.387	6.00	7.99	5.89

Unite are log_{10} 23SrDNA copies per one centimeter in intestinal contents

that the disturbed ecological niche might be induced the inflammation of the gastrointestinal tract and aggravation condition. The Enterococcus counts were re-growed to normal density after 6 days postinfection. This demonstrates that residual Enterococcus surviving the immunization can re-grow, outcompete SE and thereby re-establish colonization resistance.

CONCLUSION

This research proved that the pigeon could reduced the adherence of Enterococcus to the enteric epithelium after artificially infected by SE. In order to prevent the infection of SE, researchers may supply Enterococcus *in vitro* to adjust the counts in intestinal tract and inhibit the infection of SE.

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