Detection of intestinal pathogenic *Klebsiella pneumoniae* from fecal samples of *Giant Panda* by Polymerase Chain Reaction

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It was necessary to develop simple and reproducible diagnostic tools for endangered species, and their associated pathogens. *Klebsiella pneumoniae* was the main pathogenic bacterium leading to intestinal disease within Giant Panda (*Ailuropoda melanoleuca*). A noninvasive method for the specific detection of *K. pneumoniae* in Giant Pandas' feces was developed and evaluated in this study. The 368-bp *phoE* gene was selected for specificity, and 2 types of *K. pneumoniae* strains in conjunction with 16 non-*K. pneumoniae* strains were evaluated. The Key step of noninvasive sampling procedure was to remove the inhibitors of Polymerase Chain Reaction (PCR), which was based on that silica powders could bind pathogen DNA at the conditions of high concentration of kalium iodide and neutral pH. Before PCR cycle, the bound DNA was washed with 80% ethanol and eluted with a diluted buffer. To validate the PCR assay, an experiment was performed with both artificially contaminated and natural fecal matter. The results indicated that the method could detect specifically *K. pneumoniae* from Giant Pandas' feces with considerable sensitivity.

Keywords: PCR; *Ailuropoda melanoleuca*; noninvasive technique; diagnosis.

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Introduction

Wild Giant Pandas are endemic to China and one of the rarest animals in the world. As a member of the endangered species list [1], their population health and survival conditions were the foci of many studies. There were several reasons why Giant Pandas were threatened with extinction. Research had shown that the primary reason was due to intestinal disease [2, 3]. *Klebsiella pneumoniae* was one of the chief pathogenic bacteria causing intestinal disease of Giant Pandas [4]. There were two typical symptoms exhibited by Giant Pandas infected with *K. pneumoniae*. Enteritis was the most typical one. Giant Pandas suffering from it crouched by stockyard despondently, along with inappetence, even exhibiting mucus and blood in diarrhea. *K. pneumoniae* infection could also lead to a second common symptom of infected Giant Pandas, blood poisoning, which appeared initially with the same symptoms as enteritis. However, if efficacious diagnosis and therapy were not given in time, pathological changes could take place in the heart, lung, spleen, liver and lymph etc. The overall effect would be the death of the Giant Pandas [4, 5]. In recent years, the incidence of *K. pneumoniae* had increased significantly in feces of Giant Pandas suffered from intestinal diseases [4, 6]. Therefore, this pathogenic microbe was considered fearful menace to the

survival of Giant Pandas, especially to the cub and adolescent Giant Pandas [2, 5, 6]. Knowledge of domesticated Giant Pandas told us that it was very difficult to well treat the infected individual after they had become seriously infected. It was urgent to develop a rapid effective diagnostic method in order to decrease the negative effect *K. pneumoniae* on the wild Giant Pandas population. In order to reach such a goal, we conducted a study on the basis of correlative principles and successful experiences using PCR method as it was applied to human disease detection. Because wild Giant Pandas had definite den domain, this method presented a brief and credible approach to intestinal disease diagnosis for both domestic and wild Giant Pandas.

It was impossible to sample by conventional methods such as destructive and invasive sampling due to the vulnerable survival conditions of endangered species [7]. This problem, however, had been solved to a great extent by means of noninvasive sampling based on the advancement of molecular biological techniques in recent years, e.g. urine, hair and feces sampling [8; 9]. With the combination of traditional fecal analysis and molecular technology, we could detect the pathogenic bacteria by extracting DNA from animal feces without disturbing or even meeting them. But, it was difficult to obtain DNA pure enough to manipulate molecular procedure such as PCR from feces [10], because there were many components existing in feces and they could inhibit the polymerase activity or change ion concentration of the reaction system. DNA purification from feces was the vital process in developing a systemic method to detect pathogen from Giant Pandas' feces.

Materials and Methods

Bacterial strains:

The bacterial strains used for assay development and assessment of primer specificities were listed in Tables 1. A total of 2 types *K. pneumoniae* strains (from American Type Culture Collection (ATCC), Manassas, VA, USA) and 16 non-*K. pneumoniae* strains (2 from American Type Culture Collection (ATCC), Manassas, VA, USA; 11 from Centers for Medical Culture Collection (CMCC), Beijing, China; 3 Isolated from samples) were used. The 16 non-*K. pneumonia* strains were all the main opportunistic pathogenic bacteria which potentially were found in Panda gut. All bacterial strains were obtained from the Centers for Medical Culture Collection (CMCC), Beijing, China. When the specificity of the assay was evaluated, the negative control contained all bacterial species but *K. pneumoniae*. Artificially contaminated samples and natural fecal samples were also tested through conventional culture methods with serological confirmation and the VITEK test system (BioMerieux SA, France).

Sample collection:

Fresh natural feces of Giant Pandas were collected from Louguantai Wild Animal Protection and Breeding Center in China. All samples were collected using aseptic techniques, transported to the laboratory on ice, and stored overnight at 4°C before analysis. Bacterial pure cultures were serially diluted and confirmed by plating on standard plate count agar in triplicate. Suspension of bacteria was made by putting sterile redistilled water into bacterial pure culture. Artificially contaminated fecal samples were prepared by mixing the suspension of bacteria with fresh natural fecal samples of Giant Pandas.

Preparation of silica dioxide suspension:

There were large numbers of complex components existing in Giant Pandas' feces, which potentially inhibited the PCR reaction activity. It was known that silica dioxide could adsorb DNA specifically at the conditions of high concentration of kalium iodide in a neutral pH environment. Therefore, in this study, a silica dioxide based method was used to purify DNA from Giant Pandas' feces samples, in preparation for PCR amplification. The process

Table 1. Bacteria used for assay development and assessment of primer specificities

Bacteria	Source	Reference No.	No. of strains
Klebsiella pneumoniae	ATCC[a]	13883, 35657	2
Salmonella ssp	CMCC[b]	50825C, 50781E, 50798F	3
Listeria.monocytohenes	ATCC	19118	1
Staphyloccocus aureus	CMCC	26003	1
EHEC O157：H7	Isolated	25113, Isolated in Shannxi hospital	1
Yersinia enterocolitica	CMCC	52215, 52217, 52219	3
Vibrio parahemolyticus	CMCC	20516	1
Enterobacter sakazak	Isolated	23602, Isolated from feed/silage	1
Clostridium perfringens	Isolated	23116, Isolated from sewage	1
Shigella flexneri	CMCC	51409	1
Campylobacter jejuni	ATCC	33252	1
Pseudomonas aeruginosa	CMCC	15442, 10211	2

[a] ATCC—American Type Culture Collection, Manassas, USA.
[b] CMCC—Centers for Medical Culture Collection, Beijing, China.

was as follows: put 120mg of silica dioxide into 1,000 µl sterile redistilled water, and then stirred it vigorously overnight. After 24 hours of settlement at room temperature, the supernatant was removed, and then re-suspended the precipitation in 1,000 µl of 8 M sodium iodide, stirred it fiercely, allowed it to resettle for 5 hours, and sterilized it in a high pressure. The prepared silica dioxide suspension was stored in dark at 4°C for use [11, 12].

Templates extraction from artificially contaminated samples and natural samples:

The steps of extracting template DNA from artificially contaminated fecal samples or natural fecal samples were as follows: a gram of feces samples were placed in a centrifuge tube, centrifuged at 4,000 × g for 5 min in order to get rid of excremental residue, supernatant was gathered in 1.5 ml Eppendorf tube, centrifuged again at 10,000 × g for another 5 min for getting thalli together, then upper water was discard, the precipitation was suspended in 100 µl sterile redistilled water, then disposed it by lysis buffer which included components of 100 µg proteinase K, 400 µl 0.05 mol/L EDTA, 400 µl 1 mol/L Tris (pH 8.0), 20 µl NP-40 and 900 µl sterile redistilled water, it was then followed by incubation at 55°C for 30 min for digesting, the samples were boiled in a water bath for 5 min for inactivation, after adding 50 µl silica dioxide suspension into the tube, the tubes were mixed and adsorbed for 10 min. Subsequently, the precipitation was washed twice in 400 µl 80% ethanol and then centrifuged at 10,000 × g for 5 min, the supernatant was then removed using a pipette, the silica-bound bacterium DNA was dried at 37°C for 10 min, the DNA was then eluted by adding 100 µl sterile redistilled water and incubation at 56°C for 5 min for desorption, centrifuged the samples at 10,000 × g. finally, the supernatant containing bacterium DNA was transferred into a new 1.5 ml Eppendorf tube as template [6, 13, 14]. As a control, the traditional phenol/chloroform extraction method was used in another sample at the same time [15].

Sensitivity studies

Sensitivity studies were performed with *K. pneumoniae* ATCC 13883 contaminated fecal sample in order to determine the lower

detection limit of the PCR assay. DNA was prepared from a dilution series with a range from 10^0 to 10^4 copies/g fecal samples. To rule out false positives, one un-inoculated aliquot was used as a negative control in every experiment.

PCR amplification and product detection

Specific Primers for *K. pneumoniae* (Forward: 5-TGG CCC GCG CCC AGG GTT CGA AA-3 and Reverse: 5-GAT GTC GTC ATC GTT GAT GCC GAG-3) were designed to amplify a 368 bp fragment of the outer membrane phosphoporin protein E (*phoE*) gene. Primers were synthesized by Shanghai Sangon Co. China. Other reagents used in this study included Proteinase K, Taq DNA polymerase, 4× dNTP, Marker, Agarose, Tris, Ethidium Bromide, and so on. (All from Hua Mei Co., Ltd. China).

PCR analysis was performed in the Eppendorf gradient PCR (Eppendorf, Hamburg, Germany). The components of PCR reaction system were templates (10 ng/ml), buffers (1×), Primers (0.2 µmol/L), Taq DNA polymerase (1 U/µl), dNTPs (200 µmol/L). Total reaction volume was 30 µl. Optimization of cycling conditions resulted in initial denaturation for 5 min at 95°C followed by 35 cycles each consisting of denaturation (1 min, 94°C), annealing (1 min, 55°C) and elongation (1 min, 72°C). An incubation step of 5 min at 72°C was added.

The product of amplification was detected by Agarose gel Electrophoresis. Electrophoresis was done on 1.2% agarose gels contained 0.5 µg/ml ethidium bromide in which 5 µL reaction products were loaded with 1 µL loading buffer. Gels were run at 100 V for 30 min, visualized on a UV transilluminator and photograph by UVP imaging system (Beckman Coulter, Indianapolis, IN, USA).

Results and Discussion

Specificity and sensitivity of this assay:

To evaluate the specificity of primers, extracted DNA from pure cultures of 2 types *K. pneumoniae* strains and 16 non-*K. pneumoniae* strains were examined as template. As expected, 2 types *K. pneumoniae* strains were positive and produced 368 bp amplification bands. No bands appear in the lane of all non-*K. pneumoniae* strains (result not shown). Specificity of PCR products was also identified by direct sequencing. The sequencing work was carried out by GenScript Co., Ltd. China. Analysis of sequence data and homology comparisons were performed by using the BLAST online at the NCBI homepage (http://www.ncbi.nlm.nih.gov) (result not shown).

The PCR products sequence was correlated with *phoE* gene partial region of *K. pneumoniae* with GenBank accession number EF197995.1 (99% homology). The result showed that the primer was specific for *K. pneumoniae.*

The result of sensitivity test was shown in Figure 1. The detection limit for this assay was approximately 10 copies/gram fecal samples.

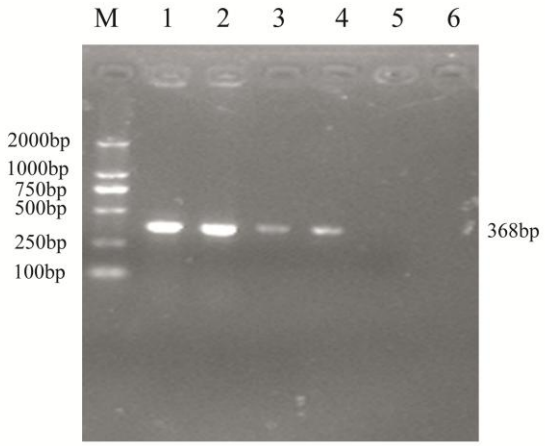

Figure 1. Sensitivity of the PCR assay with *K. pneumoniae* 10-fold serial Dilutions from 10^4 copies to 10^0 copies. Lane M: DNA marker (D2000); lane 1: 10^4 copies/g fecal samples, lane 2: 10^3 copies/g fecal samples, lane 3: 10^2 copies/g fecal samples, lane 4: 10^1 copies/g fecal samples, lane 5: 10^0 copies/g fecal samples, lane 6: NTC, negative control (no bacteria).

Detection of *K. pneumoniae* in fecal samples: Artificially contaminated fecal samples and natural fecal samples were detected individually by the above methods. At the same time, the negative control, the positive control, and the fecal samples treated by the traditional DNA purification method were all detected. The PCR amplification results were shown in Figure 2.

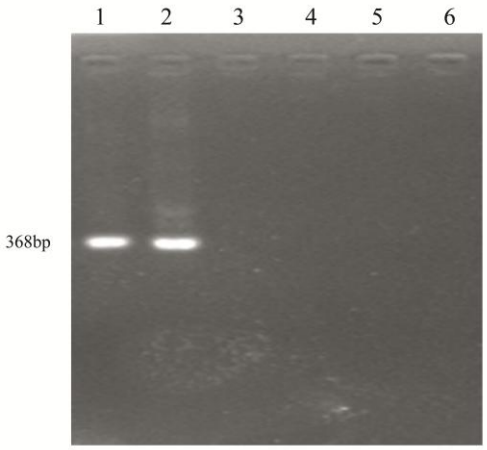

Figure 2. Result of feces sample detection by PCR. Lane 1: positive control, lane 2: SiO_2 method treated artificially contaminated fecal samples, lane 3: traditional method treated artificially contaminated fecal samples, lane 4: SiO_2 method treated natural fecal samples, lane 5: traditional method treated natural fecal samples, lane 6: negative control.

As expected, in lane 2, there was a clear, specific amplification band as well as the positive control one in lane 1. Again as expected, No band was found in lane 3 for traditional method treated artificially contaminated fecal samples with *K. pneumoniae*, the same as the negative control one in lane 6. Natural fecal samples were analyzed for the presence of *K. pneumoniae* by SiO_2 method and traditional method. No band was found either in lane 4 for SiO_2 method or in lane 5 for traditional method. All natural fecal samples analyzed were negative for *K. pneumoniae* by both PCR assay and standard culture method. No false positive or false negative results were detected. This confirms that the method could be employed for the detection of *K. pneumoniae* pathogens from feces of Giant Pandas.

DNA tests such as PCR analysis allowed the use of different kinds of samples [16, 17]. However, it was not possible to sample by conventions method such as destructive or invasive sampling due to the vulnerable survival conditions of endangered species [7]. In contrast to the blood or muscle collection method, Feces sampling was a more satisfactory method. Without disturbing or even observing animals, DNA tests could be performed by extracting DNA from animal feces [9]. Though, from feces, it was difficult to obtain pure enough DNA to manipulate molecular procedure. The problem was traced to the presence of co-purified excremental substances like bilirubin, bile salt, thalli albumen, multiplex amylase and several heavy metal ion which inhibited the polymerase activity or change ion concentration of the reaction system [10].

The result of fecal sample detection by PCR shown in Figure 2 that no band was found in lane 3 indicated the undoubted presence of an inhibitor to PCR in the fecal samples and demonstrated traditional DNA purification method incapacity to remove them. In the traditional DNA purification method, phenol / chloroform was generally used to remove protein components so as to achieve the purpose of purifying DNA. When the PCR inhibitor was water-soluble substances, phenol, chloroform, even ethanol didn't have the ability to effectively remove it. It would be precipitated along with the DNA. False negative results were the product of this phenomenon. In order to acquire accurate data, it was very important to eliminate PCR-inhibiting impurities present in DNA samples from feces. To overcome such difficult, a silica dioxide based method to purify DNA from giant Panda's feces was developed in this study. The silica powders could bind pathogen DNA at a certain conditions. The method shortened the period of diagnosis and enhanced the efficiency of

positive detection. And all operations were in the identical tube, which prevented samples from potential contamination. The homothetic clear bands presented in lane 2 and lane 1 are compellent evidences. Specific amplification band only appeared in K. pneumoniae strains and the capability of detection was 10 copies/g fecal samples. The result suggested that both sensitivity and specificity of the above method were generally high.

In summary, this work described a simple, rapid, specific and credible method to detect *K. pneumoniae* from Giant Pandas' feces. Furthermore such a method could be designed as kits for the disease diagnosis of Giant Pandas and other wild animals suffering from infection of *K. pneumoniae*. It was well known that PCR method had high sensitivity [18, 19]. This assay could also be used in the disease preventive surveillance such as periodic detection to pathogenic microbe in stockyard surroundings for conservation of Chinese endemic Giant Pandas.

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