Simultaneous detection of multiple fly-borne bacterial pathogenic microorganisms by the reverse line blot hybridization assay

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Research article

Keywords: Reverse line blot; fly-borne; hybridisation assay; bacterial pathogens; probes

Posted Date: February 25th, 2020

DOI: https://doi.org/10.21203/rs.2.21814/v2

Abstract

Background: As a widespread health pest, flies can carry more than 100 kinds of path ogenic microbes to threat human health, resulting in a wide range of disease infection and transmission. The aim of this study was to develop a sensitive, reliable and rapid method for the simultaneous detection of multiple fly-borne bacterial pathogenic microorganisms, in order to effectively prevent and control fly-borne bacterial diseases. Results: PCR-RLB method could directly and accurately detect fly-borne bacteria species corresponding of 7 species-specific probes. The sensitivity of PCR-RLB was significantly higher (about 100 times) than that of PCR. At the same time, the membrane binding oligonucleotide species-specific probes prepared in RLB detection technology can be reused for detection of bacteria after washing with 0.5 M EDTA, which greatly improves the detection efficiency. This method was used to detect the intestinal pathogens carried by flies in four different areas of Lanzhou city. In 106 groups of samples from different areas, The numbers of carrying seven bacterial strains were 2 (S. aureus), 52 (S. flexneri), 0 (A. caviae), 3% (V. vulnificus), 56 (S. enterica), 1 (P. vulgaris) and 33 (Y. enterocolitica), respectively. Their proportions of 7 bacterial strains carried by houseflies were 1.23% (S. aureus), 32.1% (S. flexneri), 0% (A. caviae), 1.85% (V. vulnificus), 34.57% (S. enterica), 0.62% (P. vulgaris) and 20.37% (Y. enterocolitica), respectively. The results showed that there was a high correlation between the bacteria carrying rate of flies and the health environment in urban areas. S. enterica , S. flexneri and Y. enterocolitica accounted for the overwhelming majority of the seven pathogenic strains carried by houseflies. This indicated that houseflies played an important role in the transmission of intestinal infectious diseases. S. aureus and V. vulnificus were carried by houseflies near the hospital area indicates that hospitals should do well in killing and controlling flies and further strengthen the prevention and control of fly-borne bacterial diseases. Conclusion: The developed PCR-RLB assay have potential clinical application in the simultaneous detection of fly-borne bacterial species.

Background

As an important vector for insect-vector diseases, flies carry or disseminate a variety of bacterial pathogenic microorganisms to cause human diarrhea, food poisoning and various bacterial diseases such as cholera, bacteremia, tuberculosis, anthrax, and the like [1-8]. There are a wide variety of flies, among which only a few species are the most common in the human family and around the farm, including housefly, stomoxys calcitrans, lucilia sericata, sarcophagidae and so on. Flies feed and reproduce in animal feces, organic wastes and carcasses, being one of the important threats to human health [9-18]. On February 24, 2005, the Science Times reported that flies spread of *Enterohemorrhagic E. coli* and *avian influenza* in Japanese. In China, flies are also included in the key prevention and control target of insect-borne diseases.

Insect-borne bacterial disease detection is mainly based on traditional bacterial culture and isolation. Identification of each strain takes at least a week or so. This method is time and labor-consuming and has strict requirements on the laboratory environment, which leads to the isolation and cultivation of bacteria cannot be carried out in areas without large laboratories, and seriously affect the prevention and

treatment of insect-borne diseases. The rapid and efficient detection of insect-borne bacterial diseases has become a hot field in the prevention and control of insect-borne diseases. Reverse line blot (RLB) is a sensitive and high-throughput detection method, which can simultaneously detect various pathogenic microorganisms carried by insects. Its essence is the combination of PCR product single chain and species-specific probe to determine the difference of the amplified sequence. PCR-RLB technology has high sensitivity and specificity. It can distinguish various strains of mixed infection, and even identify species. So it was widely used in the detection of various diseases, such as Kaufhold et al. (1994) for the first time to use PCR-RLB in serotype identification of *streptococcus* [19]; O'Sullivan et al. (2011) used PCR-RLB technology to analyze the drug resistant strains of *Staphylococcus aureus* [20]; Nijhof et al. (2005) applied this method to analyze four species of *Taylor* in Africa [21].

The aim of this study was to develop an optimized PCR-RLB hybridization assay which could simultaneously detect 7 kinds of bacteria including *Staphylococcus aureus*, *Shigella flexneri*, *Aeromonas caviae*, *Vibrio vulnificus*, *Salmonella enterica subsp. entericaserovar typhimurium*, *Proteus vulgaris* and *Yersinia enterocolitica subsp. enterocolitica* efficiently and quickly. And this method was used to gather information on the bacteria carried by houseflies randomly obtained from four different environment including residential area, slaughterhouse, garbage and hospital in Lanzhou, China. The effects of different urban environments on the fly-borne bacteria were preliminarily discussed.

Methods

Standard bacterial strains

The standard strains for this experiment focus on the main intestinal pathogens. Standard strains of 7 bacterial species selected to develop the assay were purchased from Shanghai Bioplus Biotech Co., Ltd (Shanghai, China), and their sources are shown in Table 1. The standard strains were identified by VITEK 2 Compact automatic bacterial identification and analysis system from the microbiology laboratory of the Quarantine Service (Gansu Provincial Center for Disease Control and Prevention (GSCDC), Lanzhou, Gansu province, China).

Collection and treatment of housefly samples

A total of 1060 houseflies were randomly collected from four different environments in Lanzhou of China, including residential area (n=380), slaughterhouse (n=330), garbage transfer station (n=200), near hospital (n=150) from 2016 to 2017. Ten houseflies per group were packed into autoclaved triangular flasks, 10 ml physiological saline was added, and washed by shaking for 10 min for subsequent DNA extraction.

DNA extraction

DNA was extracted from the overnight cultures of the bacteria using DNA extraction kit for Gram-negative bacteria (ABT) according to the manufacturer's instructions. The extracted DNA was stored at -20 ºC until

the subsequent analysis. Briefly, 1 ml of the overnight bacterial culture was centrifuged for 5 min at 10000 rpm, the supernatant was discarded. 1 ml physiological saline was added in the precipitate, and the above mixture was shocked to disperse bacteria and then centrifuged for 5 min at 10000 rpm, the supernatant was discarded. 200 μl of sterilized ddH_2O was then added, mixed thoroughly and the supernatant was discarded after centrifuging for 3 min at 13000 rpm. After adding 50 μl nucleic acid extract into the bacteria precipitate, mixed thoroughly and centrifuged instantaneously, the hanging wall liquid was flung to the bottom of the EP tube. The EP tube containing the bacteria solution was heated in water bath at 100 ºC for 10 min, and then centrifuged for 10 min at 13000 rpm, the supernatant was used as the DNA template in subsequent amplification experiments.

Primer and probe design

The 16S RNA was found out to be highly conservative, according to the literature [22] and the GenBank database. The sequence alignment of the ribosome 16S RNA of 7 bacterial species (*S. aureus*, *S. flexneri*, *A. caviae*, *V. vulnificus*, *S. enterica*, *P. vulgaris*, *Y. enterocolitica*) was carried out. Universal primer (RLB-F, RLB-R) for PCR amplification of genomic DNA samples used in PCR-RLB hybridization assay, species-specific probe and universal probe (Catch-all) were designed using DNAStar and Primer premier software. To test for theoretical specificity, all the primers and probes used were aligned with the sequence databases of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLASTn). Universal primers were labelled at the 5'-end with biotin to allow PCR products to be detected by hybridisation with a streptavidin–peroxidase substrate in the RLB assay. All probes were labelled at the 5'-end with an amine group to facilitate covalent linkage to nylon membranes and to allow membranes to be stripped and reused repeatedly. The primers and probes were synthetized by Sangon Biotech Company, China (Table 2).

PCR amplification

Genomic DNA (of the standard strains or samples) was added to a reaction mixture (final volume of 25 μl) containing 40M of both primer RLB-F and RLB-R. PCR amplification was performed in an automatic DNA thermocycler (Eppendorf). The reaction was incubated at 94 ºC for 5 min to denature genomic DNA and the thermal cycle reaction programme was: 30 s at 94 ºC, 30 s at 63 ºC and 45 s at 72 ºC for 35 cycles with a final extension step of 72 ºC for 10 min. Samples were held at 12 ºC until analysis.

RLB hybridization

The RLB protocol was performed as described previously [23]. Briefly, a Biodyne C blotting membrane (BNBCH5R, Pall BioSupport) was activated at room temperature by incubating in 16% EDAC (E7750, Sigma) for 10 min, then washed in distilled water, and placed in a MN45 miniblotter (FZB, Germany). Species-specific oligonucleotide probes were diluted to different concentrations (25, 50, 100, 200, 500, 800, 1000 μM) in 500 mM $NaHCO_3$ (pH 8.4), added to the miniblotter slots, and incubated for 2 min. Then, the membrane was incubated in 100 mM NaOH for 10 min and rinsed with demineralized water at 60 °C for 5 min in 2 × SSPE/0.1 % SDS. The membrane was then placed perpendicular to the probe

orientation in the miniblotter. Twenty microliters of each PCR product was diluted in 2×SSPE with SDS 10% w/v to a final volume of 150 µl, heated to 99 °C for 10 min, and then cooled immediately on ice. The denatured PCR products were then added to the slots in the miniblotter and incubated for 60 min at 60 °C, and the membrane was washed twice at 60 °C for 10 min in 2×SSPE with SDS 0.5 %. Additionally, the membrane was treated at 42 °C for 60 min with peroxidase-labeled streptavidin diluted 1:4000 in 2×SSPE/0.5 % SDS and washed twice at 42 °C for 10 min in 2×SSPE/0.5 % SDS and twice at room temperature for 5 min in 2×SSPE. Finally, chemiluminescence detection was performed according to standard procedures (Amersham).

Specificity and sensitivity of RLB

For specificity studies, DNA was extracted from standard strains (Table 1) using a DNA extraction kit for Gram-negative bacteria (ABT) according to the manufacturer's instructions, and was tested against all probe sets.

To assess RLB sensitivity, the genomic DNA content of the standard strains was determined by nucleic acid concentration meter (NanoDrop ND-2000). Serial ten-fold dilutions of genomic DNA (starting at 100 ng/µl) were prepared into 10^{-1}-10^{-12} in distilled water and then used as template for the RLB sensitivity analysis.

Results

Selection of probes and primers

A pair of primers (RLB-F, RLB-R) 20–23 bp in length was designed for amplification of all standard strains, with amplicon sizes in the range 104–1270 bp. The result of PCR amplification was shown in Fig. 1. The size of PCR amplification products is about 1100 bp, consisted with the amplicon sizes of the designed primers, which reveals the designed primers can successfully amplified the target sequence fragments.

Twelve different oligonucleotide probes directed against 7 bacterial species were designed. In addition, a universal probe targeting the 16S rRNA gene was used as a control. *A. caviae*-1 probe, *P. vulgaris*-2 probe did not show any cross-reaction with 7 standard strains. In addition, *S. aureus*-1, *S. flexneri*-1, *Y. enterocolitica*-1 probe simultaneously identified two bacterial species. Therefore these five oligonucleotide probes cannot be used in PCR-RLB experiments. The finally selected oligonucleotide probes were *S. aureus*-2, *S. flexneri*-2, *A. caviae*-2, *V. vulnificus*, *S. enterica*, *P. vulgaris*-1, *Y. enterocolitica*-2 probe (Fig. 2).

Initial evaluation experiments revealed that the optimal primer concentration of RLB-F and RLB-R was 50 µM; the optimal probe concentration was 50 µM for Catch-all, *S.aureus*-2 and *S.flexneri*-2 probe, and 100 µM for other selected probes (Table 2).

Specificity of RLB

All selected probes bound only to their respective target sequence, resulting in the recognition of individual bacterial species. The nucleotide probes did not show any cross-reaction with water used as a blank control. The catch-all probe specifically detected any standard strains present. Each standard strain was identified by two oligonucleotide probes: the catch-all probe and species-specific probes for either 7 bacterial species (Fig. 3).

Sensitivity of RLB

The RLB assay is capable of detecting about 10^{-8} ng/μL (*S. aureus*), 10^{-8} ng/μL (*S. flexneri*), 10^{-6} ng/μL (*A.caviae*), 10^{-6} ng/μL (*V. vulnificus*), 10^{-11} ng/μL (*S. enterica*), 10^{-6} ng/μL (*P. vulgaris*), and 10^{-11} ng/μL (*Y. enterocolitica*) (Fig. 4). To test the capacity of the developed PCR-RLB assay to detect 7 bacterial species, subjected to PCR and subsequently evaluated. The sensitivity of traditional PCR was shown in Fig. 5: 10^{-4} ng/μL (*S. aureus*), 10^{-2} ng/μL (*S. flexneri*), 10^{-4} ng/μL (*A.caviae*), 10^{-4} ng/μL (*V. vulnificus*), 10^{-7} ng/μL (*S. enterica*), 10^{-4} ng/μL (*P. vulgaris*), 10^{-3} ng/μL (*Y. enterocolitica*). The results showed that the sensitivity of PCR-RLB was significantly higher (about 100 times) than that of PCR.

Simultaneous detection of 7 fly-borne bacterial pathogenic microorganisms by PCR-RLB

In summary, 1060 houseflies (divided into 106 groups) from four different environments in Lanzhou of China were detected for 7 fly-borne bacterial pathogenic microorganisms by using RLB. Compared with traditional PCR, the PCR-RLB method can accurately detect different bacterial species corresponding to species-specific oligonucleotide probes, and the unknown bacterial species can be detected by universal probes. The results shown in Fig. 6 can not only clearly display the bacterial carrying status of samples, but also analyze the carrying and carrier rate of bacteria corresponding to different probes. The detail of the analysis results was shown in Fig. 7 and Table 3.

The proportions of 7 pathogenic species carried by all samples from four different environments were 1.23% (*S. aureus*), 32.1% (*S. flexneri*), 0% (*A. caviae*), 1.85% (*V. vulnificus*), 34.57% (*S. enterica*), 0.62% (*P. vulgaris*), 20.37% (*Y. enterocolitica*), respectively. In general, *A. caviae* is not carried by all samples. *S. flexneri, S. enteric, Y. enterocolitica* are the most prevalent pathogenic species carried by houseflies. It is worth noting that houseflies near hospital carried almost all these pathogenic species except *A. caviae*. The carrier rates of *Y. enterocolitica* carried by houseflies in residential areas and garbage transfer station are the highest. And *S. flexneri* carried by houseflies is the most popular in slaughterhouse and hospital.

Discussion

In all kinds of bacterial detection experiments, the first thing is to clarify the source of bacterial strain, and confirm that bacterial strain did not mutate, so all the standard strains used in this study have been identified. Sequence alignment of 16S RNA gene sequences of 7 standard bacterial strains with clear background was carried out, universal primers and species-specific oligonucleotide probes were

successfully designed. The target sequences of all bacterial strains were successfully amplified by using universal primers for PCR amplification. And species-specific probes (*S.aureus*-2, *S.flexneri*-2, *A.caviae*-2, *V. vulnificus, S. enteric, P. vulgaris*-1, *Y. enterocolitica*-2) aimed at the target gene sequences were successfully screened out. A simultaneous detection method of 7 bacteria species by PCR-RLB was successfully established. At the same time, the membrane binding oligonucleotide species-specific probes prepared in RLB detection technology can be reused for detection of bacteria after washing with 0.5 M EDTA, which greatly improves the detection efficiency. The sensitivity of the PCR amplification products with different concentration prepared by serial ten-fold dilutions was tested. The results showed that the sensitivity of PCR-RLB was significantly higher (about 100 times) than that of PCR, which is consistent with literature reports [23-29].

From 2004 to 2010, the incidence of enteric diseases such as bacillary dysentery, typhoid, hepatitis A and other infectious diarrhea in Lanzhou still accounted for 24.19% - 44.46% of the total incidence of Category A, B and C infectious diseases. Especially in recent years, intestinal infectious diseases occur frequently, and several bacterial food poisoning cases have been reported, which shows that intestinal infectious diseases and bacterial food poisoning are still the focus of prevention and control in Lanzhou city. Therefore, this developed rapid detection of intestinal infectious bacteria carried by flies is an important measure to prevent and control infectious diseases. In this study, four areas of Lanzhou City were selected to collect flies to make clear the position of flies as a biological vector in the transmission of intestinal infectious diseases in Lanzhou. Firstly, it is necessary to study whether the bacteria species carried by flies in residential areas have a direct relationship with intestinal infectious diseases. Secondly, waste transfer station and slaughterhouse where fly breeding is considered. In addition, the slaughterhouse is also the base of meat food for all residents in Lanzhou. If flies in this place carry a large number of intestinal pathogens, the disease may be transmitted to residents through meat pollution. Flies near the hospital are selected for testing to make sure whether the hospital disinfection and sterilization work is perfect. PCR-RLB method could directly and accurately detect fly-borne bacteria species corresponding of 7 species-specific probes. In 106 groups of samples from four areas, the numbers of samples carrying seven different bacterial strains were 2 (*S. aureus*), 52 (*S. flexneri*), 0 (*A. caviae*), 3% (*V. vulnificus*), 56 (*S. enterica*), 1 (*P. vulgaris*) and 33 (*Y. enterocolitica*), respectively. Their proportions of 7 bacterial strains carried by houseflies were 1.23% (*S. aureus*), 32.1% (*S. flexneri*), 0% (*A. caviae*), 1.85% (*V. vulnificus*), 34.57% (*S. enterica*), 0.62% (*P. vulgaris*) and 20.37% (*Y. enterocolitica*), respectively. RLB test results show that flies are important vector of intestinal infectious diseases and bacterial food poisoning, and its harmfulness cannot be ignored. Flies from residential area and slaughterhouse both carried a large number of intestinal pathogens such as *S. flexneri, S. enterica* and *Y. enterocolitica*, further proving that flies were important carriers of intestinal infectious diseases. The above pathogens can cause diarrhea, vomiting and other symptoms of intestinal pathogens. Flies carrying these pathogenic bacteria can pollute food, tableware, daily necessities and the surrounding environment. Through contact or ingestion, the risk of intestinal infectious diseases and bacterial food poisoning increases. Flies near hospital not only carried common intestinal pathogens, but also carried *S. aureus* and *V.vulnificus.* So hospitals should do well in killing and controlling flies and further strengthen

the prevention and control of fly-borne bacterial diseases. The above results indicate that we should improve the urban environment and block the source of vector transmission, so as to effectively control the spread of insect-borne diseases.

Conclusion

The aim of this study was to develop a sensitive, reliable and rapid method for the simultaneous detection of multiple fly-borne bacterial pathogenic microorganisms, in order to effectively prevent and control fly-borne bacterial diseases. A PCR-based reverse line blot (PCR-RLB) hybridisation assay was developed. All species-specific probes designed for the RLB hybridised with amplified DNA only from the corresponding species. The sensitivity of the PCR amplification products with different concentration prepared by serial ten-fold dilutions was tested, and the results showed that the sensitivity of PCR-RLB was significantly higher (about 100 times) than that of PCR. This method was then used to gather information on the bacteria carried by houseflies randomly obtained from four different environments in Lanzhou, China. The effects of different urban environments on the fly-borne bacteria were preliminarily discussed. The RLB assay appeared to have potential clinical application in the simultaneous detection of fly-borne bacterial species.

Abbreviations

PCR:polymerase chain reaction; PCR-RLB:PCR-based reverse line blot hybridization; EDTA:ethylene diamine tetraacetic acid.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

The project was supported by the fund of Gansu Agricultural University (GAU-KYQD-2017RCZX-11, GAU-XKJS-2018-073), Gansu Province natural sciences fund (18JR3RA167), Key research and development projects in gansu province (18YF1FA080), Lanzhou science and technology plan project (2017-4-103), Biotechnology Project of Gansu Agriculture and Animal Husbandry Department (GNSW-2014-9), National Natural Science Regional Foundation Project (CN) (31460659) and Open Fund of State Key Laboratory of Pathogenic Biology of Livestock Epidemic Diseases (SKLVEB2014KFKT002). No information has been provided by the authors concerning the existence or absence of conflicting or dual interests.

Authors' contributions

YM, JL, and HG conceived and designed the experiments. ML and YT were instrumental in the acquisition of data. YM, HG and XS analysed the data and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

Pro. Jianxun Luo, Dr. Qingli Niu, and Dr. Qiaoyun Ren are thanked for their support of and assistance with the experiment and the collection of specimens.

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Figures

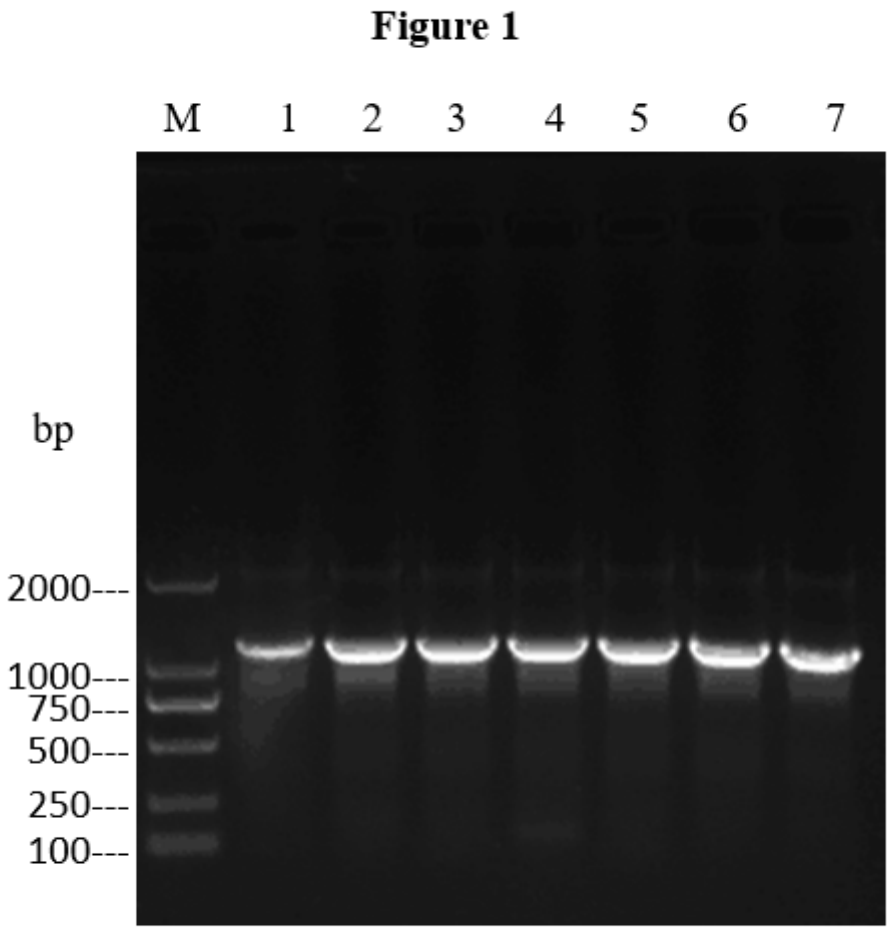

Figure 1

The PCR amplication result of 7 standard strains. Lane: M, DL2000 DNA marker; lane 1-7: S. aureus, S. flexneri, A. caviae, V. vulnificus, S. enterica, P. vulgaris, Y. enterocolitica.

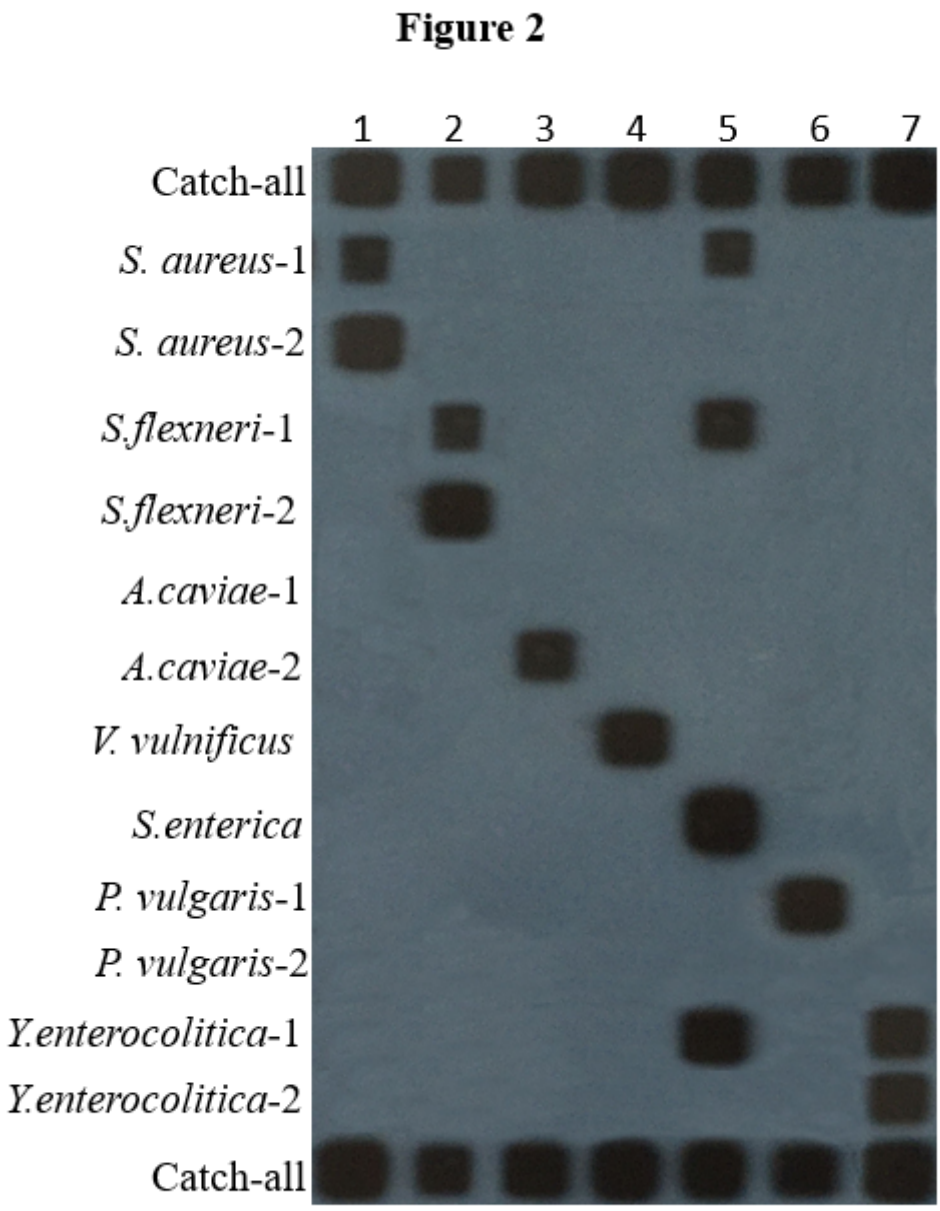

Figure 2

The selection of probe. Oligonucleotides probes are applied in horizontal rows and PCR products are applied in vertical lanes. Lanes: PCR product hybridization with probe. 1 to 7 indicate PCR products of 7 standard strains (S. aureus, S. flexneri, A. caviae, V. vulnificus, S. enterica, P. vulgaris and Y. enterocolitica, respectively); Rows 1 and 14 indicate catch-all, 2-13 indicate S. aureus-1, S. aureus-2, S. flexneri-1, S. flexneri-2, A. caviae-1, A. caviae-2, V. vulnificus, S.enterica, P. vulgaris-1, P. vulgaris-2, Y. enterocolitica-1, Y. enterocolitica-2 probe, respectively.

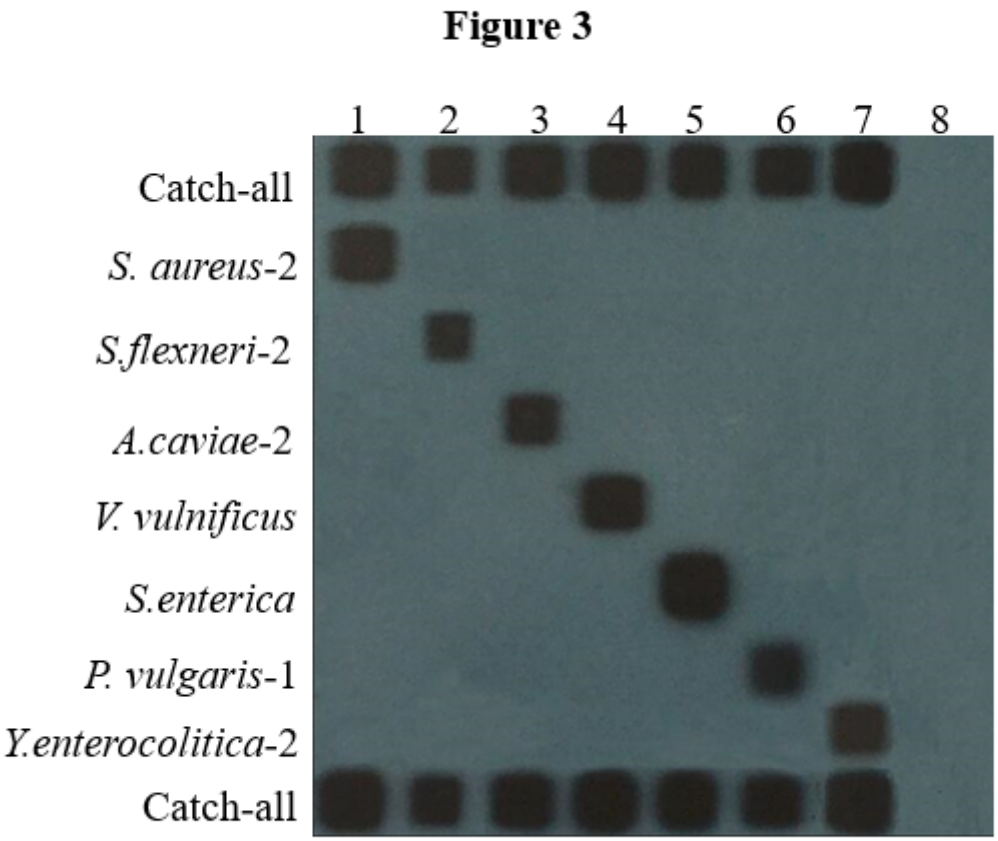

Figure 3

PCR-RLB specificity experiment results of seven strains. Oligonucleotides probes are applied in horizontal rows and PCR products are applied in vertical lanes. Lanes: PCR product hybridization with probe. 1 to 7 indicate PCR products of 7 standard strains (S. aureus, S. flexneri, A. caviae, V. vulnificus, S. enterica, P. vulgaris and Y. enterocolitica, respectively), 8 indicates blank control; Rows 1 and 9 indicate catch-all, 2-8 indicate S. aureus-2, S. flexneri-2, A. caviae-2, V. vulnificus, S.enterica, P. vulgaris-1, Y. enterocolitica-2 probe, respectively.

Figure 4

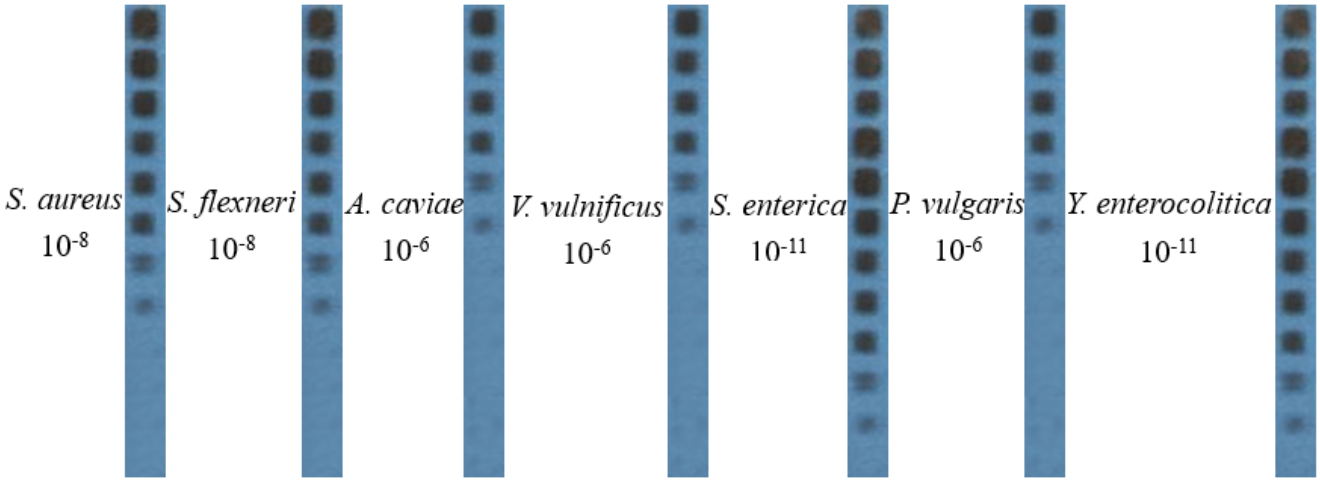

Figure 4

PCR-RLB sensitivity experiment results of seven strains. Oligonucleotides probes are applied in vertical lanes, and serial ten-fold dilutions of genomic DNA (starting at 100 ng/µl) prepared into 10-1-10-12 in distilled water are applied in horizontal rows 1-12, respectively. The assay detected concentration of about 10-8 ng/µL (S. aureus), 10-8 ng/µL (S. flexneri), 10-6 ng/µL (A.caviae), 10-6 ng/µL (V. vulnificus), 10-11 ng/µL (S. enterica), 10-6 ng/µL (P. vulgaris), and 10-11 ng/µL (Y. enterocolitica).

Figure 5

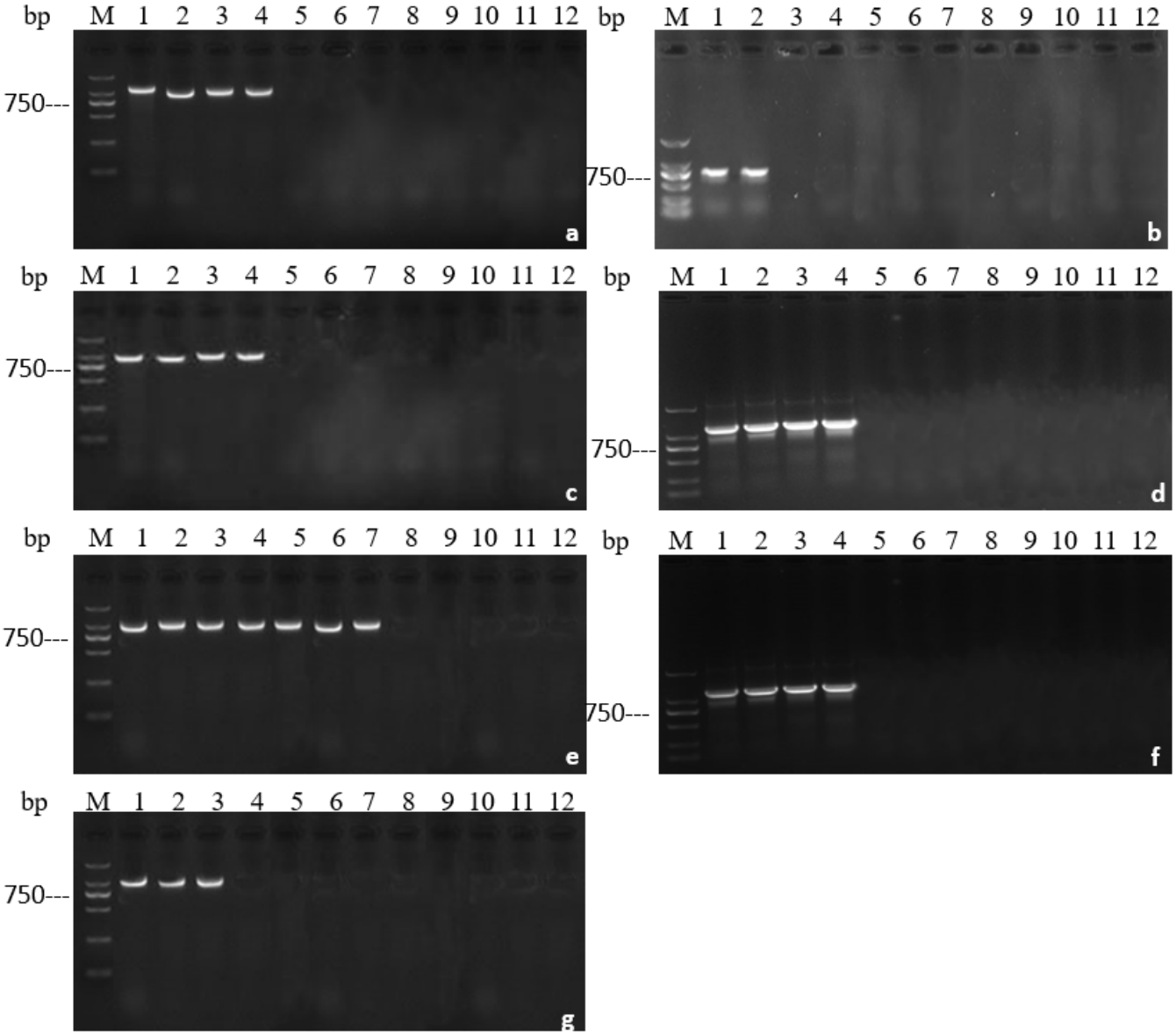

Figure 5

PCR sensitivity experiment results of seven strains. Serial ten-fold dilutions of genomic DNA (starting at 100 ng/μl) were prepared into 10-1-10-12 in distilled water and amplified by PCR, and the results are shown in lanes 1-12, respectively; lane M, DL2000 DNA marker. (a)-(g) show the detection of 7 standard strains (S. aureus, S. flexneri, A. caviae, V. vulnificus, S. enterica, P. vulgaris and Y. enterocolitica, respectively) by PCR.

Figure 6

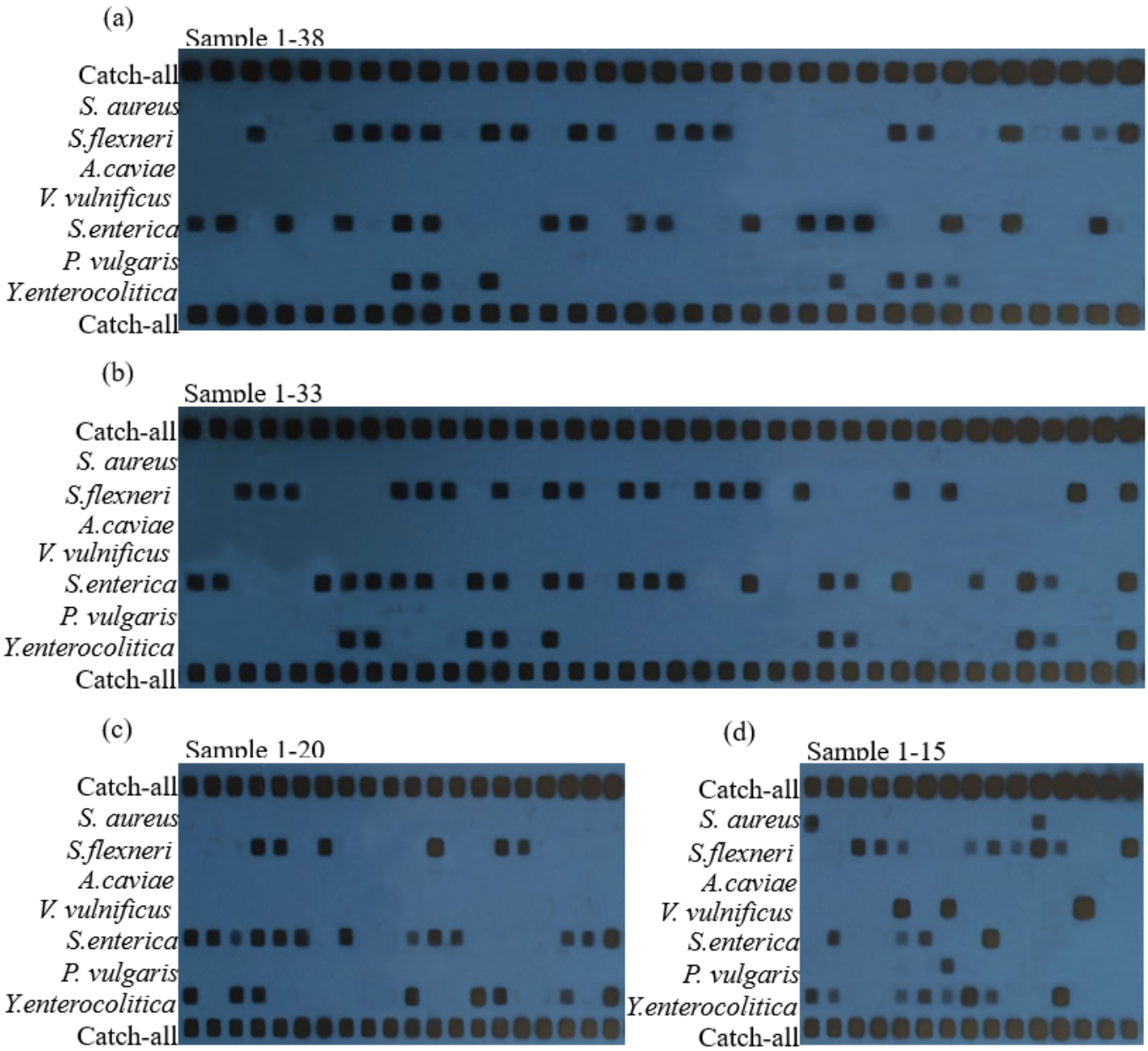

Figure 6

The detection of bacteria carried by 106 groups (10 samples/group) houseflies from four different environments in Lanzhou. Oligonucleotides probes are indicated on the y-axis, and samples are indicated on the x-axis. (a) The assay detected samples collected from residential area, lanes 1-38 are indicated 38 groups of samples, respectively. (b) The assay detected samples collected from Slaughterhouse, lanes 1-33 are indicated 33 groups of samples, respectively. (c) The assay detected samples collected from garbage transfer station, lanes 1-20 are indicated 20 groups of samples, respectively. (d) The assay detected samples collected near hospital, lanes 1-15 are indicated 15 groups of samples, respectively.

Figure 7

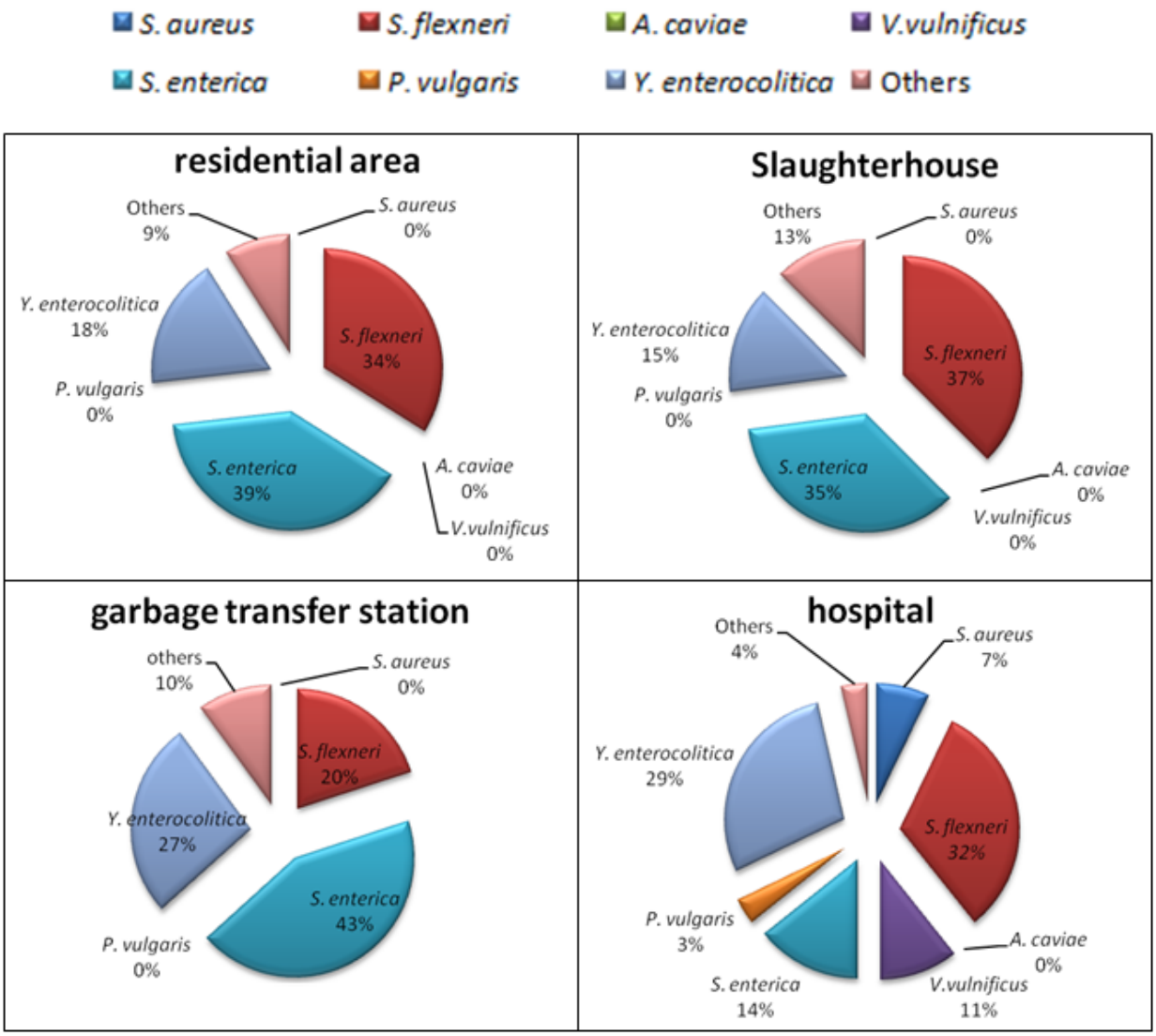

Figure 7

The statistical result of detection bacteria carried by houseflies collected from four different environments of Lanzhou using PCR-RLB method.