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Evaluation of the Efficiency of a Congo-Red Uptake Technique for Detection and Isolation of Plasmid-Bearing (Pyv) *Yersinia pestis* KIM5 in Retail Raw Ground Beef and Pork

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Abstract

Knowledge of the presence of *Yersinia pestis* in food may be useful in the event that *Y. pestis* is used in a bioterrorism attack in the food supply. However, there are no reports on the enrichment and detection of *Y. pestis* in food. Congo red-uptake (CR-uptake) associated with the virulence plasmid was used to identify *Y. pestis* inoculated at various levels into ground beef (RGB) and pork (RGP) in fresh, cold-stored, and freeze-stored conditions. *Y. pestis* KIM5 was detected in fresh and refrigerated-stored sterile and non-sterile RGB and RGP when inoculated at 10^8, 10^6 or 10^3 CFU/g, but was not detected at 10^2 or 10^1 CFU/g level of inoculum. In RGB and RGP freeze-stored *Y. pestis* for 6 days, was detected only when inoculated into the meats at 10^6 to 10^8 CFU/g. Thus, the CR-method is suitable for detection of moderate levels of *Y. pestis* contamination without enrichment. Furthermore, the method is suitable for the isolation of *Y. pestis* from ground meat.

Keywords: *Y. Pestis;* Ground meat; Efficiency; Detection; Isolation

Introduction

After the terrorist attack in the United States in 2001, there was the realization that foods may be vulnerable to bioterrorism by intentional contamination with known food pathogens, as well as with biological agents that are not usually considered threats to the food supply. This awareness has led to an increased need for screening methods for highly infectious pathogens such as *Yersinia pestis* that may be intentionally inoculated into the food supply. The potential use of this pathogen in food as a biological weapon could cause pneumonic plague in the U.S. population and the widespread loss of human life [1-4]. Furthermore, multidrug-resistant strains of *Y. pestis* may cause difficult to treat infections [5]. In this regard, risk assessors are interested in knowing the fate of *Y. pestis* for scenarios where bulk foods could be contaminated.

Yersinia pestis is primarily a rodent pathogen, with humans being an accidental host if bitten by an infected rat flea [6]. The ingestion of *Y. pestis* as a mechanism of transmission is now well recognized. Animal models indicate that *Y. pestis* can be transmitted by the oral route. Rust [7] reported that infections were seen when rats and mice consumed organ parts from experimental animals that had died from plague infection. Post-mortem analysis showed that a large number of *Y. pestis* were present in infected rats and mice that had died from plague. Furthermore, Butler [8] showed that mice infected orally by drinking water seeded with 10^8 *Y. pestis* per milliliter died after 3 days. Transmission can be achieved by handling or consumption of raw or cooked meat products prepared from animals infected with *Y. pestis* [9-12].

Retail Ground Beef (RGB) and Pork (RGP) were selected as foods for inoculation in the current study for the following reasons: ground beef is produced in large production lots totaling ca. 7.5 billion pounds (3.4 billion kilograms) in the U.S., and, if contaminated with a pathogen, could affect the health of numerous consumers. In ground beef processing plants, combo bins with capacities of ~2,000 pounds are filled with beef trim, blended with other combo bins and ground (American Meat Institute Fact Sheet, 2004). The resulting ground product is distributed throughout wholesale and retail markets in chubs and as hamburger patties (8.2 billion) (National Cattlemen's Beef Association, 2005). Pork products such as ground pork, pork sausage, chops, and ribs, are produced on a large scale. The United States Department of Agriculture estimates the per capita ground pork consumption in the U.S. at 65.5 pounds per year (National Agricultural Statistics Service, USDA, 2011). The resulting pork products are distributed throughout wholesale and retail markets.

Previously, we reported a differential phenotype expression method for detection and isolation of *Y. pestis* based on Congo red-uptake (CR-uptake) associated with the virulence plasmid (pYV) [13]. In our recent studies, we demonstrated that CR-uptake allowed detection and isolation of *Y. pestis* during its growth in both RGB [14] and RGP [15]. The CR-uptake method was successfully used to study pYV stability during its growth in those ground meat products [14-16] and was also used in development of a procedure to monitor the presence of pYV under different culture conditions. Since there is no report on quantitative data on detection levels of *Y. pestis* in ground beef and pork; we investigated whether CR-uptake allowed the detection of low levels of *Y. pestis.* Both sterile and non-sterile RGB and RGP were used in this study in order to fully assess the potential of this CR-uptake identification method. It is necessary to know the effect of the endogenous microflora on the ability to detect the pathogen in RGB and RGP containing the endogenous microflora. Furthermore, during slaughter, processing, transport, and storage for various time periods, ground beef and pork are refrigerated at retail, and then are often refrigerated or frozen by the consumer at –20°C for extended storage. Hence, we evaluated the effect of refrigeration and freezing on the detection and isolation of *Y. pestis* in RGB and RGP at low levels

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using the CR-uptake technique. The information obtained will help the evaluation of the efficiency of the CR-uptake technique on the detention and isolation of *Y. pestis* in beef and ground pork.

Yersinia pestis expresses an envelope glycoprotein called Fraction 1 (F1) antigen only at temperatures >33°C. Serum antibodies to F1 are measured using passive hemagglutination assays (PHA). High titers of antibody along with correlating symptoms, such as buboes, generally indicate a positive diagnosis. Further testing may include X-rays of the lung to check for presence of pneumonic plague, examination of sputum, and lymph node biopsies. A short-term inactivated vaccine against *Y. pestis* has existed since the mid-19th century. Though its efficacy has never been precisely measured, field data does show that it lessens incidence and severity of disease resulting from animal transmission. The vaccine is recommended only for laboratory or field workers working with the pathogen, or persons (e.g. Peace Corps volunteers) residing temporarily in rural areas containing the enzootic plague in both human and animal carriers. Though death rates for untreated cases usually approach 100%, antibiotics can be a very effective treatment against the plague. In some instances, the vaccine will only ameliorate illness, in which case a rigorous treatment of antibiotics is administered. *Yersinia pestis* is very susceptible to streptomycin and chloramphenicol; however, concomitant therapy is highly recommended to avoid shock resulting from the lysis of high numbers of Gram-negative cells and the induction of a severe inflammatory response.

Materials and Methods

Ground meat

The RGB with 7% fat was purchased at a local supermarket. The RGP (3.2 mm pieces, 20% fat) was made from fresh pork steak cubes purchased from a local slaughter and processing plant. For studies using sterile meat, RGB and RGP were divided into 90-g portions, vacuum-sealed (1k mbar) in filter double-sealed Stomacher bags (Koch Industries, Kansas City, Missouri), frozen at -80°C, shipped to a commercial irradiation facility (Food Technology Service, Inc., Mulberry, FL) and irradiated at –80°C to a dose of 42 kGy for 7 h. After sterilization, frozen samples were shipped to the Eastern Regional Research Center and stored at –80°C until experimentation. The sterility of samples was periodically verified by preparing a 10-fold dilution of RGB and RGP in 1% peptone water (PW, Becton-Dickinson, Sparks, MD), plating 0.1 ml onto brain heart infusion agar (BHA; Becton-Dickinson) and examining plates for colonies after 24 and 48 h incubation at 37°C. For non-sterile product studies, RGB and RGP were also placed in 90-g portions in vacuum-sealed (1k mbar) double-sealed stomacher bags and stored at –80°C until experimentation.

Plasmid-bearing Yersinia pestis strain and maintenance

Yersinia pestis KIM5, a derivative of strain KIM (Kurdistan Iranian Man), which lacks the chromosomally-encoded pigmentation (Pgm$^-$) locus, but contains all three virulence plasmids [pYV (70.3-kb, YOPs, type III secretion system), pFra/pMT1 (96.2-kb, murine toxin: phospholipase, F1 capsule-like antigen), and pCP1/pPst/pPla (9.6-kb, plasminogen activator) [17] was used in this study. This strain is conditionally virulent (a conditional mutant is only infectious if inoculated intravenously) and can be used in a BL2 laboratory facility [17]. This strain is well characterized, and has been extensively used in our laboratory, as well as by other investigators, to study the microbiology and molecular pathogenesis of this bacterium [12-17]. Since we used RGB and RGP with its natural microflora to evaluate the sensitivity and efficiency of detection and isolation of *Y. pestis* KIM5, it was necessary to use an antibiotic resistant strain of *Y. pestis* KIM5 to detect the organism in RGP. A rifampicin (rif) resistant *Y. pestis* KIM5 (rif-*Y. pestis* KIM5) was isolated on tryptic soy agar (TSA; Becton-Dickinson, Franklin Lakes, NJ) containing 100 µg of rif (Sigma Chemicals St. Louis, MO) per ml and incubated at 28°C for 72 h to prevent plasmid loss as described by Bhaduri and Phillips [13]. The rif-*Y. pestis* KIM5 strain was maintained at –80°C in brain heart infusion (BHI; Becton-Dickinson) broth containing 20% glycerol until ready to use. Storage of cultures, preparation of inocula, and incubation conditions have been described previously. Due to the unstable nature of pYV, the presence of pYV in starting cultures of *Y. pestis* KIM5 and rif-*Y. pestis* KIM5 in each experiment was confirmed by low calcium response (Lcr), Congo red (CR) binding, and a PCR assay targeting a key regulatory gene, *virF*, present on pYV.

Preparation of media

Calcium-deficient Congo red (CR) magnesium oxalate agar (CRMOX) was prepared by adding 20% D-galactose (Sigma Chemical Co., St. Louis, MO), 0.25 M sodium oxalate (Sigma), 0.25 M magnesium chloride (Sigma) and 1% CR (Sigma) to tryptic soy agar (TSA) (Becton-Dickinson,), as described by Bhaduri et al. The pYV-bearing *Y. pestis* KIM5 appeared as red pinpoint colonies (CR-uptake) on CRMOX after incubation at 37°C for 48 h showing Lcr and CR-uptake phenotypes encoded by pYV. The pYV-bearing rif-*Y. pestis* KIM5 was detected by plating on CRMOX containing 100 µg per ml of rif (rif-CRMOX) where pYV-bearing rif-*Y. pestis* KIM5 appeared as red pinpoint colonies as mentioned above.

Preparation of inocula

Both pYV-bearing *Y. pestis* KIM5 and pYV-bearing rif- *Y. pestis* KIM5 were grown in BHI broth for 48 h at 28°C with shaking at 120 rpm to a population density of approximately 10^9 colony-forming units (CFU) per ml.

Inoculation of RGB and RGP

Sterile RGB and RGP and non-sterile RGB and RGP were thawed for 2-3 h at room temperature (RT) and then maintained at 4°C until they were used for experiments. Prior to inoculating, the four types of ground meat samples were equilibrated at RT followed by inoculation with Y. pestis. Five-gram portions of each ground meat were placed in puncture proof Stomacher bags (A. J. Seward, London, UK) with a sterile spoon. Both pYV-bearing *Y. pestis* KIM5 and pYV-bearing rif-*Y. pestis* KIM5 cultures were added individually after dilution with 1% PW to the respective ground meat bags to yield levels of 10^8, 10^6, 10^3, 10^2, and 10^1 CFU/g. The inocula were hand-massaged into the ground beef in the Stomacher bag for ~30 sec, followed by stomaching (Model Bag Mixer 400, Interscience Inc., Weymouth, MA) the sample for 2 min at RT. To detect *Y. pestis* in freshly contaminated ground meat, the samples were immediately diluted with 1% PW in 10-fold serial dilutions, and 0.05 ml was surface plated on CRMOX for sterilized RGB and RGP and on rif-CRMOX for non-sterile RGB and RGP. The plates were incubated for 48 h at 37°C. The zero time counts of the inoculated ground meat were determined before refrigeration and freezing. To evaluate the effect of refrigeration and freezing on detection by the CR-uptake method, the inoculated samples were kept at 4°C and at -20°C for 2 days and 6 days, respectively, and then the samples were processed as described above. Two trials from separate batches of sterilized RGB and RGP and non-sterilized RGB and RGP were conducted for each temperature, with triplicate samples per time point and triplicate plating of each sample after serial dilution.

Bacterial enumeration

Red pin point *Y. pestis* KIM5 and rif-*Y. pestis* KIM5 colonies appearing on agar plates were counted using a ProtoCOL colony counter with version 3.15.630 software (Protocol PC Model 66000, Synoptics Ltd., UK). Data were transferred to an Excel® spreadsheet (Microsoft Corp., Redmond, WA).

Data analyses

Colony count data were transformed into efficiency values by the following formula:

$$\%\ Efficiency = \frac{Number\ of\ cells\ (CFU/gm)\ detected\ in\ ground\ meat}{Total\ number\ of\ cells\ (CFU/gm)\ inoculated\ in\ ground\ meat} \times 100$$

Efficiency scores for both RGB and RGP were analyzed to determine the effects of sterilization, inoculum size, and storage treatment and their two way interactions. This was performed by analysis of variance by assuming that the three-way interaction was negligible, and thus it was used as an error term. Non-significant main effects and two-way interactions were pooled with the error term to obtain a parsimonious model. Multiple comparison inferences were made using the Bonferroni LSD technique at the p=0.05 significance level.

Microbial counting is useful in the basic sciences and is used determine the number of bacteria present for physiological or biochemical studies. For example, if one knows the number of bacteria present in a culture then one can calculate the amount of protein or DNA that can be isolated from that population. Microbial enumeration is also routinely used in the areas of public health. Food or water microbiologists test food, milk or water for the numbers of microbial pathogens to determine if these products are safe for human consumption.

Results and Discussion

In the present study, the level of efficiency of CR-uptake to identify *Y. pestis* was evaluated using artificially contaminated sterilized and non-sterilized RGB and RGP at zero time (freshly inoculated) and after refrigeration at 4°C for 2 days and freezing at -20°C.

Detection of *Y. pestis* in RGB

Sensitivity for the detection of *Y. pestis* KIM5 by the CR uptake assay decreased with the decreasing level of the organism from 10^8 to 10^3 CFU/g in fresh samples of both sterilized and non-sterilized RGB from 91 to 50% and 85 to 44%, respectively. The efficiency of detection was slightly reduced in non-sterilized RGB under this condition at the level 10^8 and 10^6 CFU/g. Refrigeration at 4°C for 2 days did not affect the efficiency of detection for sterilized samples (100 to 53%) with the decreasing level of the organism from 10^8 to 10^3 CFU/g, and these results were similar to those of the fresh samples. However, the efficiency of detection was reduced in non-sterilized RGB when stored at 4°C compared to the fresh samples at the level 10^8 (72% efficiency) and 10^6 (56% efficiency) CFU/g of inoculum.

At 10^3 contamination level the efficiency of detection with 2 days storage at 4°C in non-sterilized RGB was reduced by 75.00% in comparison to the fresh sample and 79.25% compared to the sterilized RGB. This may be due to presence of endogenous bacteria present in the non-sterilized RGB. The endogenous bacteria on the back-ground mask the red pinpoint colonies. At the level of contamination of 10^2 and 10^1 CFU/g, *Y. pestis* KIM5 was not detected in both sterilized and non-sterilized RGB in fresh and refrigerated samples under the same conditions. *Yersinia pestis* KIM5 was detected only at 10^8 and 10^6 contamination level in both sterilized and non-sterilized RGB under freeze-stored conditions (6 days at -20°C). There was no significant difference in efficiency of detection between both RGB and RGP.

Figure 1: Microscopic structure of *Yersinia pestis*

Detection of *Y. pestis* in RGP

The data in Figure 1 show the efficiency of CR-uptake for the detection of *Y. pestis* KIM5 in sterilized and non-sterile RGP. *Yersinia pestis* KIM5 was detected from 10^8 to 10^3 CFU/g level of inoculum in fresh and refrigeration conditions. The sensitivity of this method was reduced by 20.22% at 10^8 (for fresh vs. cold-stored samples) and by 13.26% at 10^6 CFU/g inoculum level but was similar with a level of contamination of 10^3 CFU/g at 4°C storage for 2 days in non-sterilized samples. There was also a reduction of efficiency in the fresh sample at 10^3 CFU/g inoculum level. Again, in RGP, only at higher levels (10^8 and 10^6 CFU/g) could the pathogen be detected under freeze-stored conditions. Similar to RGB, the pathogen was not detectable at inoculum levels of 10^2 and 10^1 CFU/g in RGP. There was an interaction between inoculum level and storage treatment; however, there was no evidence of a significant sterilization effect.

Conclusion

In summary, the present study is the first to show that CR-uptake can be used as a tool for detection and isolation of *Y. pestis* from ground meat. *Yersinia pestis* can survive cold and freeze stress when inoculated onto ground meat and can be detected and isolated. The method is suitable for the detection of a moderate level of contamination without enrichment. However, the organism could not be detected and isolated using inoculum levels of 10^2 and 10^1 CFU/g. Furthermore, if the ground meat is intentionally contaminated it will be with a very large number of *Y. pestis*. Also at present there is no appropriate enrichment procedure to isolate *Y. pestis* from food. The present study shows that use of the CR-uptake method will add a significant margin of safety for detection of *Y. pestis* during slaughter, processing, transport, and refrigerated storage for various time periods in RGB and RGP.

References

1. Cupp OS, Walker DE, Hillison J (2004) Agroterrorism in the U.S.: key security challenge for the 21st century. Biosecur Bioterror 2: 97-105.
2. Hamdy M, Khalafalla F, Yassien N (1990) Incidence of *Yersinia enterocolitica* in slaughtered camels. Fleischwirtsch 70: 685-686.
3. Hawley RJ, Eitzen EM (2001) Biological weapons-a primer for microbiologists. Annu Rev Microbiol 55: 235-253.

4. Thomas VI, Donald AH, John GB, Michael SA, Arthur MF, et al. (1999) Anthrax, as biological weapon. Medical and public health management. J Am Med Assoc 281: 1735-1745.

5. Marc Galimand, Annie G, Guy G, Bruno R, Suzanne C, et al. (1997) Multidrugresistance in *Yersinia pestis* mediated by transferable plasmid. N Engl J Med 337: 677-680.

6. Rust JH, Harrison DN, Marshall JD, Cavanaugh DC (1972) Susceptibility of rodents to oral plague infection: a mechanism for the persistence of plague in inter-epidemic periods. J Wildl Dis 8:127-133.

7. Butler T, Fu YS, Furman L, Almeida C, Almeida A (1982) Experimental *Yersinia pestis* infection in rodents after intragastric inoculation and ingestion of bacteria. Infect Immun 36: 1160-1167.

8. Arbaji A, Kharabsheh S, Al-Azab S, Al-Kayed M, Amr ZS, et al. (2005) A 12-case outbreak of pharyngeal plague following the consumption of camel meat, in north-eastern Jordan. Ann Trop Med Parasitiol 99: 789-793.

9. Bin Saeed AA, Al-Hamdan NA, Fontainee RE (2005) Plague from eating raw camel liver. Emerg Infect Dis 11: 1456-1457.

10. Christie AB, Chen TH, Elberg SS (1980) Plague in camels and goats: their role in human epidemics. J Infect Dis 141: 724-726.

11. Leslie T, Whitehouse CA, Yingst S, Baldwin C, Kakar F, et al. (2011) Outbreak of gastroenteritis caused by *Yersinia pestis* in Afghanistan. Epidemiol. Infec 139: 728-735.

12. Bhaduri S, Sommers CH (2008) Detection of *Yersinia pestis* by comparison of virulence plasmid (PYV/PCD)-associated phenotypes in *Yersinia* species. J Food Safety 28: 453-466.

13. Bhaduri S (2010) Effect of fat in ground beef on the growth and virulence plasmid (PYV) stability in *Yersinia pestis*. Intl J Food Microbiol 136: 372-375.

14. Bhaduri S, Phillips JG (2013) Growth of a PYV-bearing *Yersinia pestis* Kim5 in retail raw ground pork. Foodborne Pathogens and Diseases 10: 467-471.

15. Bhaduri S, Phillips JG (2011) Growth model of a plasmid-bearing virulent strain of *Yersinia pseudotuberculosis* in raw ground beef. Zoonoses and Public Health 58: 77-84.

16. Brubaker RR (2006) *Yersinia pestis* and bubonic plague. New York.

17. Bearden SW, Perry RD (2008) Laboratory Maintenance and Characterization of *Yersinia pestis*. Current Protocols in Microbiology 11: 5B.1.1-5B.1.13.

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