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Comparison of Common Enrichment Methods for Recovery of *Yersinia Enterocolitica* from Artificially Inoculated Swine Feed Samples

Joo-Sung Kim* and F. A. Draughon
The University of Tennessee, Food Safety Center of Excellence, Department of Food Science and Technology, Knoxville, TN 37996, USA
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ABSTRACT - Five different enrichment methods were studied to find an optimal method to recover *Yersinia enterocolitica* from swine feed samples. When the recovery of *Y. enterocolitica* GER-C (serotype O:3) strain was studied at 1000 CFU/g feed, phosphate-buffered saline (PBS) enrichment at 4°C and PBS plus sorbitol and bile salts (PSB) enrichment at 4°C and 21°C were not effective (< 22%). In contrast, both irgasan-ticarcillin-potassium chlorate (ITC) and tryptic soy broth plus polymyxin B sulfate and novobiocin (TSBPN) enrichment methods showed a full recovery (100%) at 100-1000 CFU/g feed. At 10 CFU/g feed, both ITC and TSBPN methods still recovered the strain (> 50%). In recovery of ATCC 9610 (serotype O:8) strain, TSBPN method was more sensitive than any other methods ($P < 0.05$) at 1000 CFU/g feed. Using TSBPN method, the strain was still recovered at 100 CFU/g feed, but not at 10 CFU/g feed. With its sensitivity and relatively simple recipe, TSBPN was most desirable method to recover *Y. enterocolitica* from swine feed samples.

Key words : *Yersinia enterocolitica*, enrichment, swine, feed

Yersinia enterocolitica is a zoonotic bacterial pathogen highly associated with pigs and is assumed to be transmitted to human mainly through pork[1]. It is a gram-negative, facultatively anaerobic, and psychrotrophic bacterium. Main symptoms of human infection include fever, abdominal pain, and diarrhea[2].

Isolation of *Y. enterocolitica* from swine farm environment is highly relevant to understanding the prevalence and epidemiology of *Y. enterocolitica* in swine farm environment[3]. However, swine farm samples tend to have a high background of microflora[4]. It may be difficult to directly isolate *Y. enterocolitica* strains when they exist in very small numbers in the samples[5]. Thus, it is reasonable to use enrichment media to increase the number of *Y. enterocolitica* strains in samples.

Feed samples in animal farms can be contaminated by zoonotic pathogens[6-9]. These pathogens such as non-Typhi serotypes of *Salmonella enterica* in animal feed could be a source of human infection through the contamination of food animals[6]. In this study, five enrichment methods commonly used for *Y. enterocolitica* were compared to find the most sensitive enrichment method for recovery of *Y. enterocolitica* from swine feed samples.

*Correspondence to: 165 National Food Safety & Toxicology Center Michigan State University East Lansing, MI48824-1302
Tel: 517-884-2076, Fax: 517-432-2310
Email: jskim@msu.edu

Materials and Methods

Y. enterocolitica strains and the preparation of inoculum

Two *Y. enterocolitica* strains were used in this study. *Y. enterocolitica* ATCC 9610 (bioserotype 1B/O:8) was obtained from American Type Culture Collection (Manassas, VA, USA). *Y. enterocolitica* GER-C (serotype O:3) was supplied by Dr. Bhaduri (USDA, ARS, Philadelphia, PA, USA). To prepare an inoculum of *Y. enterocolitica* strains, bacterial cultures were grown in a static brain heart infusion broth at 21°C for 21-24 h to 10^8 CFU/ml. After ten fold serial dilutions in 0.85% saline solution, the bacterial suspensions were added to swine feed samples (10-25 g) to 1000, 100, and 10 CFU per gram of feed samples.

Swine feed samples

Freshly made grower finisher swine feed samples were collected from either Blount unit at Knoxville station, Tennessee Agricultural Experimental Station or Johnson Animal Research & Teaching Unit (JARTU) located in Knoxville, Tennessee, USA. Once the feed samples were transported to the lab, they were immediately stored at 4°C

before experiments. The grower finisher feeds used in this study were mostly composed of corn (72.2%) and soybean meal (19.9%), and did not contain any antibiotics.

Enrichment and selective plating

The detailed information of enrichment methods or methods with modifications used in this study is described in Table 1. All enrichment procedures were conducted in triplicate. In negative controls, the *Y. enterocolitica* strains were not included in enrichment samples. After incubation of enrichment samples, a loopful of the samples (ca.10 μl) were spread on cefsulodin-irgasan-novobiocin (CIN) agar plates (Difco, Sparks, MD21152) (3 or more plates per sample) and incubated at 30-32°C for 24 h. For irgasan-ticarcillin-potassium chlorate (ITC) enrichment at 1000 CFU/g sample, the enrichment samples were spread on Salmonella-Shigella agar (BBL, Cockeysville, MD21030) containing 1% sodium deoxycholate and 0.1% $CaCl_2$ (SSDC)[10] and the agar plates were incubated at 21°C for 24 h.

Identification of *Y. enterocolitica*

Presumptive *Y. enterocolitica* colonies grown on CIN/SSDC agar plates were studied for identification. More than 3 colonies per agar plate were tested using traditional biochemical tests: triple sugar iron agar, sucrose, rhamnose, urease, simmons citrate, o-Nitrophenyl-beta-D-galactopyranoside (ONPG), and oxidase.

Statistical analysis

A chi-square analysis (alpha=0.05) was studied to find any statistical difference over treatment (enrichment or strains) effects in recovery of *Y. enterocolitica*. The presence or absence of *Y. enterocolitica* strains on each plate was calculated as categorical data.

Table 1. Enrichment methods for *Y. enterocolitica* used in this study

Description of enrichment methods	References
PBS (90 ml) + sample (10 g) at 4°C for 5 weeks	13
PSB (225 ml)[a] + sample (25 g) at 4°C for 3 weeks	28
PSB (225 ml)[a] + sample (25 g) at 21°C for 3-4 days, then KOH post-enrichment treatment (0.12-0.23%) for 20-30 sec before plating	Modification
ITC (90 ml)[b] + sample (10 g) at 21-22°C for 24-48 h	10
TSBPN (90 ml)[c] + sample (10 g) at 18°C for 24 h	20

[a]PBS with 1% sorbitol and 0.15% bile salts.
[b]1% tryptone, 0.1% yeast extract, 6% $MgCl_2 \cdot 6H_2O$, 0.5% NaCl, 0.1% $KClO_3$, 0.001% malachite green solution, irgasan (1 μg/ml), and ticarcillin (1 μg/ml).
[c]Tryptic soy broth with polymyxin B sulfate (5 IU/ml) and novobiocin (10 μg/ml).

Results

Five different enrichment methods were compared to find the most sensitive enrichment protocol for recovery of *Y. enterocolitica* from artificially inoculated swine feed samples (Table 1). After enrichment in triplicate, a loopful of enrichment samples were spread mostly on CIN agar plates, incubated, and the presumptive colonies were studied for the confirmation of *Y. enterocolitica*. The recovery of *Y. enterocolitica* was confirmed by acid slant/acid butt with no gas in triple sugar iron agar test, positive for sucrose, urease, and ONPG tests, and negative for rhamnose, simmons citrate, and oxidase tests. No *Y. enterocolitica* strains were found in negative control samples.

When studies were conducted with *Y. enterocolitica* GER-C (serotype O:3) at 1000 CFU/g feed, the strain was fully recovered (100%) in both ITC and TSBPN methods (Fig. 1). In ITC enrichment, SSDC agar plating method did not have any significant defect in recovery rate (100%) compared to CIN method. In contrast to ITC and TSBPN methods, phosphate-buffered saline (PBS) and PBS plus sorbitol and bile salts (PSB) methods were found to be ineffective. No cells were recovered at 4°C for both PBS and PSB. PSB at 21°C with subsequent KOH treatment showed a limited recovery (22%). Accordingly, both ITC and TSBPN methods were significantly more efficient than PBS or PSB methods in recovery of *Y. enterocolitica* GER-C from swine feed samples ($P < 0.05$).

The recovery of *Y. enterocolitica* ATCC 9610 (serotype O:8) was also studied with the same enrichment methods. The strain was also fully recovered (100%) in TSBPN method, similar to *Y. enterocolitica* GER-C strain (Fig. 1). However, in contrast to *Y. enterocolitica* GER-C strain, it was poorly recovered (16%) in ITC method (Fig. 1). For *Y. enterocolitica* ATCC 9610 strain, TSBPN method was significantly more efficient than ITC method at 1000 CFU/g feed ($P < 0.05$). *Y. enterocolitica*

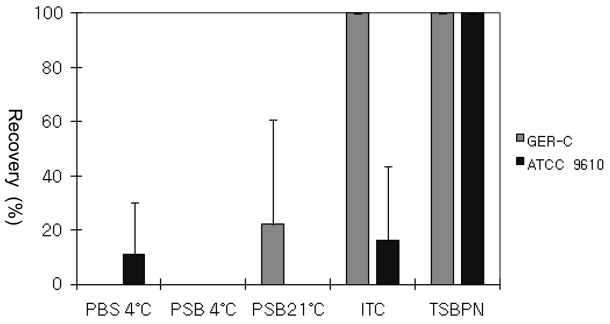

Fig. 1. Comparison of enrichment methods in recovery of *Y. enterocolitica* GER-C (O:3) and ATCC 9610 (O:8) from artificially inoculated swine feed samples at 1000 CFU/g feed. Recovery (%) was calculated as the number of *Yersinia*-positive plates divided by the number of plates tested from enrichment samples. Error bars represent standard deviations.

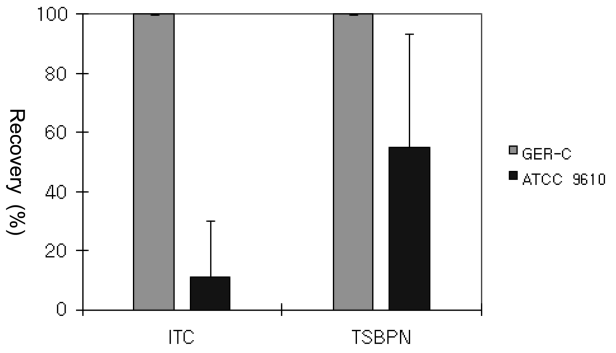

Fig. 2. Comparison of ITC to TSBPN enrichment methods in recovery of *Y. enterocolitica* GER-C (O:3) and ATCC 9610 (O:8) from artificially inoculated swine feed samples at 100 CFU/g feed. Error bars represent standard deviations.

ATCC 9610 was poorly recovered (11%) from PBS at 4°C and was not recovered from PSB at either 4 or 21°C.

Because both ITC and TSBPN methods showed satisfactory recoveries, both enrichment methods were compared at reduced level of inoculation, 100 CFU/g feed (Fig. 2). In ITC method, *Y. enterocolitica* GER-C was still fully recovered (100%) and the recovery rate of *Y. enterocolitica* GER-C was significantly higher than one of *Y. enterocolitica* ATCC 9610 (11%) ($P < 0.05$). In TSBPN method, the recovery rates of *Y. enterocolitica* GER-C and *Y. enterocolitica* ATCC 9610 were 100% and 55%, respectively. In contrast to the level of 1000 CFU/g feed, no significant difference was found between ITC and TSBPN methods in recovery of *Y. enterocolitica* ATCC 9610 at the level of 100 CFU/g feed.

Further reduced level of inoculation, 10 CFU/g feed was studied to compare the sensitivity of the ITC and TSBPN methods for *Y. enterocolitica* GER-C strain. It was still recovered in both methods and the recovery rates were 56% and 89% for ITC and TSBPN methods, respectively with no statistical difference between them. In TSBPN method, no *Y. enterocolitica* ATCC 9610 was recovered in contrast to *Y. enterocolitica* GER-C strain ($P < 0.05$).

Overall, in recovery of *Y. enterocolitica* GER-C strain, both ITC and TSBPN methods were at least 100-fold more sensitive than cold enrichment using PBS or PSB and also PSB at 21°C. In recovery of *Y. enterocolitica* ATCC 9610 strain, the TSBPN method was also more sensitive (> 10-fold) than PBS or PSB methods. Both ITC and TSBPN methods were more sensitive for *Y. enterocolitica* GER-C than *Y. enterocolitica* ATCC 9610.

Discussion

Cold enrichment using PBS or PSB has been commonly used to recover *Y. enterocolitica* from food, animal and environmental samples[11-14]. However, this study shows that cold enrichment using PBS or PSB is inefficient in contrast to ITC and TSBPN methods in recovery of *Y. enterocolitica* strains from swine feed samples. In PBS or PSB enrichment at 4°C, different incubation times (2-4 weeks for PBS; 2 and 4-5 weeks for PSB) were also studied, but no *Y. enterocolitica* strains were recovered (data not shown). Considering such a common use of cold enrichment, it is surprising that *Y. enterocolitica* strains were not recovered in cold enrichment at such a high inoculation level (1000 CFU/g feed). One possible explanation is that nutrient composition of swine feeds may not support the growth of inoculated *Y. enterocolitica* strains.

ITC enrichment method is one of most commonly used enrichment to recover *Y. enterocolitica*[15-18]. In this study, it was much more sensitive for *Y. enterocolitica* GER-C (O:3) than for *Y. enterocolitica* ATCC 9610 (O:8). Such a large difference may be due to the difference of serotypes (O:3 versus O:8). The effectiveness and selectivity of ITC enrichment for serotype O:3 was previously noticed[19].

TSBPN method, originally developed by Landgraf et al.[20], was as efficient as ITC method in recovery of *Y. enterocolitica* GER-C in this study even though it is not commonly used as much. Although Landgraf et al. used 3 day incubation, 24 h incubation was used in our study. In fact, in our study, 24 h incubation yielded higher recovery rate compared to 2-3 day incubation (data not shown). Even though TSBPN method yielded a better recovery for serotype O:3 compared to serotype O:8 in our study, its predisposition to a specific serotype is unclear[20].

Both ITC and TSBPN methods were equally most sensitive in recovery of *Y. enterocolitica* GER-C strain in this study. Because serotype O:3 appears to be the most common serotype among *Y. enterocolitica* strains in swine and its environment[3,13,21-23], these two enrichments may be ideal to monitor swine feed and farm samples. In particular, TSBPN method may be better than ITC enrichment because it performed better with *Y. enterocolitica* ATCC 9610 strain (O:8) at the relatively higher inoculation level (1000 CFU/g feed). In addition, TSBPN method is advantageous because of the relatively simple recipe. Considering that both methods were relatively insensitive to recover the O:8 strain, designing efficient enrichment to recover various serotypes equally from swine farm samples may be necessary.

Molecular techniques such as PCR have greatly improved the sensitivity in detecting pathogenic *Y. enterocolitica* from food, animal, and environmental samples[24-27]. Nevertheless, difficulties may still arise in detecting a very small number of *Y. enterocolitica* strains in the high background of microflora[5]. Thus, enrichment of samples prior to detection with molecular techniques may be still necessary to improve the detection sensitivity.

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요 약

돼지사료에 있는 여시니아균의 효율적인 검출을 위해서 다섯 종류의 증식 방법들이 비교 연구되었다. *Yersinia enterocolitica* GER-C (혈청형 O:3) 가 1000 CFU/g 사료 수준으로 들어 있을 때에 4ºC 에서의 인산완충용액과 4ºC 혹은 21ºC 에서의 솔비톨과 담즙산염을 함유한 인산완충용액은 효과적이지 못했다. 하지만, irgasan-ticarcillin-potassium chlorate (ITC) 방법과 polymyxin 과 novobiocin 을 함유한 tryptic soy broth (TSBPN) 방법은 100-1000 CFU/g 사료 수준에서 탁월한 증식효과를 보였다. ITC 와 TSBPN 방법은 10 CFU/g 사료 수준에서도 증식 및 검출 효과가 있었다. *Y. enterocolitica* ATCC 9610 (혈청형 O:8) 을 연구한 결과, 1000 CFU/g 사료 수준에서 TSBPN 증식방법이 가장 효과적이었으며, 100 CFU/g 사료 수준에서도 증식 및 검출 되었지만, 10 CFU/g 사료 수준에서는 검출되지 않았다. 검출의 민감도와 상대적으로 간단한 조성방법 면에서 볼 때, TSBPN이 돼지사료에서 *Y. enterocolitica* 를 증식, 검출하는데 있어서 가장 효과적인 방법이었다.

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