ABSTRACT

Two serotypes of Yersinia enterocolitica (0:3 and 0:8) were added to a 1% (w/v) whole egg suspension and their survival was followed during exposure to conditions which reflected those used during commercial egg washing. Trials were conducted over a range of pH (7, 9.5, 10, and 10.5) at 38 and 42°C. The potential for Yersinia growth during transport of samples was followed during exposure to conditions which reflected those used during commercial egg washing. Trials were conducted over a range of pH (7, 9.5, 10, and 10.5) at 38 and 42°C. The potential for Yersinia growth during transport of samples was followed during exposure to conditions which reflected those used during commercial egg washing. Trials were conducted over a range of pH (7, 9.5, 10, and 10.5) at 38 and 42°C.

Y. enterocolitica has several characteristics which enhance its ability to successfully contaminate processed foods. First, it is capable of growth at normal refrigeration temperatures (8). Second, and of greatest concern during egg washing, is the ability of the organism to withstand highly alkaline conditions normally capable of killing other enteric bacteria, including S. typhimurium (1,7,21).

Y. enterocolitica serotype 0:3 is the most commonly isolated serotype from cases of human clinical disease in Eastern Canada (16). In contrast, Y. enterocolitica serotype 0:8 has been isolated from frozen chicken (14) and is the most common human isolate in Western Canada and the United States (16,21,22).

In the work reported here, Y. enterocolitica serotypes 0:3 and 0:8 were examined for their ability to survive in a 1% (w/v) whole egg suspension containing detergent (synthetic egg washwater) at various temperatures from neutral to alkaline pH.

MATERIALS AND METHODS

Yersinia enterocolitica cultures

Y. enterocolitica strains 125 (serotype 0:3) and 134 (serotype 0:8) were obtained from Q.A. Laboratories Ltd., Toronto. The API 20E (API Lab Products Ltd., St. Laurent, QC) and Enterotube II (Hoffman-La Roche Ltd., Vaudreuil, QC) biochemical identification systems were inoculated with 24-h-old cultures of Y. enterocolitica strains 125 and 134. These had been taken from plates of tryptic soy broth containing 0.6% (w/v) yeast extract plus 1.5% agar (TSAYE, Difco) and suspended in sterile physiological saline solution before use. Tests were conducted in triplicate with incubation of test media at 22, 30 and 35°C for 48 h. Samples were also sent to the Laboratory Centre for Disease Control, Health and Welfare Canada, Ottawa for verification of culture identity. A combined Nilehm and Waters scheme (22) was used for biotyping (sucrose, indole and esculin, 37°C; xyllose and ornithine, 22°C; and lecithinase).

Y. enterocolitica, a potential human pathogen, has been found responsible for causing acute gastroenteritis in Canada (24) and elsewhere (19,20,22). Its isolation from water (5) and a variety of food sources, including vegetables (15), vacuum packaged beef and lamb (4), pork (8,16) and poultry (2,14,18) has demonstrated that contamination of food products may be a possible route of infection. Some isolates of Y. enterocolitica from poultry have been found to be clinically important serotypes (2,14,18). For example, Stengel (18) found serotypes 0:3 and 0:9 among isolates from 11 of 130 poultry samples positive for Y. enterocolitica. These strains were similar in phage type to human clinical isolates although virulence testing was not reported. In a study by Norberg (14) on isolation of enteropathogenic bacteria from frozen chicken, Y. enterocolitica and S. typhimurium were isolated from 24.5 and 1.2% of the 82 samples, respectively. Leistner et al. (8) isolated Y. enterocolitica from 29% of 121 samples of poultry meat and 2.3% of 88 samples of poultry feces, whereas De-
Survival of Y. enterocolitica at moderate and high pH

Sterile distilled water and the meat of one egg were combined in a sterile Waring Blender for 1 min to give a 10% (w/v) whole egg suspension. This was diluted 1:10 in sterile distilled water to yield a 1% (w/v) whole egg suspension. Portions (250 ml) of 1% whole egg suspension were brought to pH 7.0, 9.5, 10.0 or 10.5 with a 10% (w/v) filter-sterilized aqueous solution of "Best-Egg" detergent (Diversey-Wyandotte Inc. Mississauga, Ont.), and the resulting mixtures were described as synthetic egg washwater. Two 100-ml samples at each pH were measured into sterile screw-capped bottles and equilibrated to either 38 or 42°C in an oil (polyethylene glycol 200) bath (Model HB10X, Grant Instruments Ltd., Cambridge, England). Samples of the synthetic egg washwater at each pH were serially diluted in 0.1% (w/v) peptone and 0.1 or 1.0 ml portions were surface-spread in triplicate on pre-poured TSAYE plates from undiluted through 10^-2 dilutions to provide information on background levels of bacteria. When 1.0 ml was used as an inoculum, 0.4, 0.3 and 0.3 ml were surface-spread on separate plates (9 plates for each dilution).

Y. enterocolitica strains 125 and 134 were cultured separately in tryptic soy broth containing 0.6% yeast extract (TSBYE, Difco) for 48 h at 30°C. From each culture, 0.1 ml was added separately to egg washwater samples at each of the four pH values. Samples at pH 7.0 or 9.5 were taken after 0, 30, 60, 90 and 240 min. The washwater at pH 10 was tested after 0, 10, 20, 30, 60 and 240 min. The pH 10.5 water samples were analyzed 0, 5, 10, 15, 60 and 240 min after inoculation. Each sample was serially diluted in 0.1% (w/v) peptone and 0.1 or 1.0 ml portions of appropriate dilutions were surface-spread as noted above in triplicate on pre-poured TSAYE plates and incubated for 48 h at 30°C.

The study was repeated at pH 9.5, 10 and 10.5 and temperatures of 6, 10, 12 and 15°C using equal numbers of cells from both strains in a mixture. Temperatures were maintained in refrigerated incubators. Sampling was done at 0, 1, 5, 17 and 23 h, and the viability of the organisms was checked by plating on TSAYE as described above.

RESULTS

Y. enterocolitica survival

API 20E and Enterotube II biochemical analysis of the two Y. enterocolitica strains confirmed their systematic classification (data not presented). Y. enterocolitica strains 125 (0:3) and 134 (0:8) were found to be biotypes 4 and 2, respectively. Neither produced heat-stable toxin nor cytotoxin.

To test the relevance of our pH tolerance tests to the alkali procedures used to isolate Y. enterocolitica from foods (1, 2, 3), the pH of KOH solutions used by others for Y. enterocolitica isolation (0.25% to 0.45% w/v KOH) were examined and found to be ≥ 12.1.

The capacity for survival of Y. enterocolitica strains 125 and 134 in synthetic egg washwater was unaffected by temperatures of 38 or 42°C at pH values of 7.0 (Fig. 1) and 9.5 (Fig. 2). Survival varied between strains of Y. enterocolitica during exposure to thermal and alkaline stresses (Fig. 3 and 4). Inactivation of Y. enterocolitica strain 125 was observed at ≥ pH 10 and 42°C but Y. enterocolitica 134 was sensitive at both 38 and 42°C when the pH was ≥ 10. Data presented in Fig. 1-4 demonstrated that increasing the temperature from 38 to 42°C with a concomitant increase in pH from 7.0 through 10.5 substantially decreased the survival of Y. enterocolitica.

When Y. enterocolitica 125 and 134 were co-cultured in synthetic egg washwater at pH 9.5 (Fig. 5) a slight decrease in their numbers was noted at 6°C but growth...
FATE OF *Y. ENTEROCOLITICA* IN EGG WASHWATER

**Figure 4.** The survival of *Y. enterocolitica* in synthetic egg washwater at pH 10.5. Symbols as in Fig 1.

**Figure 5.** Survival of *Y. enterocolitica* 125 and 134 co-cultured in synthetic egg washwater of pH 9.5 at 6°C, (○); 10°C, (●); 12°C, (△); and 15°C, (▲).

**Figure 6.** Survival of *Y. enterocolitica* 125 and 134 co-cultured in synthetic egg washwater at pH 10.0 Symbols as in Fig 5.

**Figure 7.** Survival of *Y. enterocolitica* 125 and 134 co-cultured in synthetic egg washwater at pH 10.5. Symbols as in Fig. 5.

occurred at 10, 12 and 15°C. Washwater at pH values of 10 (Fig. 6) and 10.5 (Fig. 7) did not greatly reduce the population of co-cultured *Y. enterocolitica* at temperatures between 6 and 15°C over 24 h. However, no growth occured in experimental samples with pH ≥ 10.

**DISCUSSION**

The compound used to develop alkaline conditions in the synthetic washwater, "Best Egg", is a proprietary mixture containing a surfactant, sodium carbonate, alkaline phosphate and sodium silicate with about 0.6-0.8% available chlorine. Our analysis using Quantab chloride titrator strips (#1175, Miles Lab. Inc., Elkhart, IN) as well as the manufacturer's specifications indicated that the synthetic washwater tested at pH 10.5 initially had 10.4 ppm free chloride. Levels of chloride in pH 10.0 and 9.5 washwater were estimated to have been 2.9 and 1.9 ppm, respectively. At these low concentrations and temperatures of 38 and 42°C active chloride from the parent compound in "Best Egg", potassium dichloroisocyanurate, might be expected to have some bactericidal effect at pH 10.5 (25). However, in view of the presence of organic matter in test solutions, (concentrations which reasonably represent the situation in commercial practice), major effects on the viability of the bacteria studied would have been most likely due to the interactive effects of temperature and alkalinity since these low levels of active chloride derived from dichloroisocyanurate would be expected to become rapidly inactivated (10).
Background levels of organisms in synthetic washwater were checked and found to range between 0 and $< 6 \times 10^3$ bacteria/ml. In view of the large numbers of test bacteria inoculated at the start of experiments ($>10^6$ /ml) this low level of background contamination was not expected to cause interference.

*Y. enterocolitica* was shown to be remarkably resistant to the combined effects of thermal and alkaline stresses in synthetic egg washwater where exposures were patterned after those likely to be experienced by this organism during commercial egg washing.

Stern et al. (21) observed that at pH 10 (25°C), *Y. enterocolitica* experienced 100% inactivation within 12 h, but the organism grew when the pH was changed to 9.6 at the same temperature. When *Y. enterocolitica* was inoculated into synthetic egg washwater at pH 9.5 and exposed to temperatures of 38 and 42°C, neither growth nor substantial inactivation occurred during the 4-h test (Fig. 2). These results were similar to those seen at pH 7.0 except that cells of strain 125 increased slightly in number at 38°C (Fig. 1). In contrast, the anti-bacterial action of exposure to pH $\geq 10$ (Fig. 3 and 4) became obvious. At temperatures of 10,12 and 15°C, synthetic egg washwater supported growth of *Y. enterocolitica* at pH 9.5 (Fig. 5), but slight inactivation was observed under the same conditions at pH 10 and 10.5 (Fig. 6 and 7). Initial inactivation occurred most rapidly at the highest pH. The differential effect of pH at 9.5 and when $\geq 10$ on the survival of *Y. enterocolitica* can be attributed to an interaction of high alkalinity with thermal stress. These findings support a pH of 10 as a critical alkaline limit for the survival of *Y. enterocolitica* over a temperature range of 6°C to 42°C.

During routine country-wide surveys conducted in 1984 and reported on a quarterly basis by the Food Production and Inspection Branch of Agriculture Canada (unpublished data), between 36-40% of 2355 commercial egg washwater samples had a pH <10 while 27-38% of 1951 samples had temperatures <40°C. These observations underline the importance of regular monitoring of washwater conditions common in commercially operated egg washwater systems. Microorganisms inoculated at the start of experiments (>10<sup>6</sup> /ml) to the combined effects of thermal and alkaline stresses to the survival of *Y. enterocolitica* under the same conditions at pH 10 and 10.5 (Fig. 6). These results were similar to those seen at pH 7.0 except that cells of strain 125 increased slightly in number at 38°C (Fig. 1). In contrast, the anti-bacterial action of exposure to pH $\geq 10$ (Fig. 3 and 4) became obvious. At temperatures of 10, 12 and 15°C, synthetic egg washwater supported growth of *Y. enterocolitica* at pH 9.5 (Fig. 5), but slight inactivation was observed under the same conditions at pH 10 and 10.5 (Fig. 6 and 7). Initial inactivation occurred most rapidly at the highest pH. The differential effect of pH at 9.5 and when $\geq 10$ on the survival of *Y. enterocolitica* can be attributed to an interaction of high alkalinity with thermal stress. These findings support a pH of 10 as a critical alkaline limit for the survival of *Y. enterocolitica* over a temperature range of 6°C to 42°C.

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*Y. enterocolitica* 134 was more sensitive to the high pH of washwater than 125. (Fig. 3 and 4). Schiemann (15) found that strains of these two *Y. enterocolitica* serotypes were reversed in their sensitivity to alkaline pH at temperatures of 10, 22 and 32°C. Our work has demonstrated that *Y. enterocolitica* 125 has the ability to survive in egg washwater (synthetic) at pH 10 and 38°C, conditions common in commercially operated egg washing machines (unpublished data, 7). This resistance of *Y. enterocolitica* to alkaline conditions has proven valuable in the enumeration of *Y. enterocolitica* from mixed bacterial populations. Momentary exposure of samples to pH of $\geq 12.1$ has been used for selective isolation of *Y. enterocolitica* from foods (1, 2, 3).

Both *Y. enterocolitica* strains tolerated pH 10 (38°C) washwater better than *S. typhimurium* (6). At pH 10 and 42°C only *Y. enterocolitica* 125 was more tolerant than *S. typhimurium*. At pH 10.5 *Y. enterocolitica* 125 was still more resistant than *S. typhimurium* at 38°C. Since foodborne illness from poultry products has commonly been associated with *S. typhimurium* (23), the capability of *Y. enterocolitica* to survive in egg washwater under conditions commonly encountered at grading stations establishes it as an additional threat to health.

*Y. enterocolitica* was able to survive ionic stress more efficiently at the lower test temperatures. Leistner et al. (8) observed growth of *Y. enterocolitica* at 4°C, yet our observations in synthetic egg washwater at 6°C indicated slight inactivation. It is thought that the stress of exposure to alkaline conditions in the washwater is responsible for this difference in results. Indeed, Stern et al. (21) reported substantial inactivation of *Y. enterocolitica* in brain heart infusion broth at pH 9.6 and 3°C. It is of importance to the analysis of commercial egg washwater, that growth was observed in the mixed (two strain) culture of *Y. enterocolitica* under refrigerated conditions (Fig. 5). Since there is a delay between the time samples are taken and before their analysis at the regional laboratories, this increase in numbers of *Yersinia* at low temperatures may result in an overestimate of the true numbers of bacteria present at the time samples were taken.

*Y. enterocolitica* is not regarded as a highly competitive organism in mixed cultures (15). This is thought to result from "metabolic crowding" by faster growing antagonistic gram-negative organisms (17). However, these findings cannot be directly extrapolated to commercial egg washing due to the poor survival of most gram-negative species under the thermal (temperature $\geq 40^\circ$C) and alkaline (pH $\geq 10$) stresses common in egg washwater environments (7). It is not known whether gram-positive bacteria, which constitute most organisms in commercial egg washwater (11) where non-quaternary ammonium compounds are used (13) exert the same inhibitory influence as gram-negatives upon the growth of *Y. enterocolitica*. A study on the antagonism of gram-positive organisms toward *Y. enterocolitica* in a synthetic washwater system may help in our understanding of the potential for growth of *Y. enterocolitica* in washwater at commercially operated grading stations where water recycling is used.

**CONCLUSION**

Poultry have been shown to carry *Y. enterocolitica* in their feces (8) which is itself, a direct link with the egg production industry. Direct contamination of machinery by eggs or indirect contamination via washwater may lead to a potential health risk through the continuous contamination of washwater and subsequently eggs, from machinery (9, 11, 12, 13). The cleanliness of egg grading stations and maintenance of an anti-bacterial condition in washwater (temperature $\geq 40^\circ$C and pH $\geq 10$) play an important role in preventing *Y. enterocolitica* from contaminating marketable eggs.

This study has demonstrated that *Y. enterocolitica*, a potential human pathogen should be considered as a bacterium of concern by the egg production industry.
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