CNU Journal of Agricultural Science Vol. 41, No. 2, pp. 135-139, June 2014
DOI: http://dx.doi.org/10.7744/cnujas.2014.41.2.135

Establishment of a linear regression equation for quantification of beta-hemolytic *Escherichia coli* in different media and survival of hemolytic *Escherichia coli* after blending with three different media

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Received on March 25 2014, revised on 19 May 2014, accepted on 16 June 2014

Abstract : Pathogenic *E. coli* associated post-weaning diarrhea (PWD) and edema disease are common diseases in commercially-housed weanling pigs. An enterotoxigenic *E. coli* (ETEC) oral challenge model has been used to mimic the physiological responses observed in commercial conditions. However, an oral challenge procedure has two major limitations: (1) the ETEC cell density is unknown at the point of oral inoculation, and (2) blending ETEC with traditional TSB (trypticase soy broth) is not palatable and hence decreases acceptability by piglets. Therefore, the purposes of this study were to (1) establish a regression equation that can be used for estimation of ETEC concentration in dilution media using the spectrophotometric measurement of cell density; and (2) examine survival of ETEC after blending either with TSB, sweetener or dextrose. A strain of ETEC (serogroup beta-hemolytic *E. coli* O149; K91; F4; toxins LT, STa, STb) was grown in TSB for 3.5 hours, centrifuged, the supernatant was discarded, and the ETEC pellet was then blended either with TSB (100 mL), sweetener (60 mL TSB + 40 mL fruit flavored concentrate), or dextrose (50 mL TSB + 50 mL dextrose; 0.5g/mL dextrose). Cell density was measured using the colorimetric method and also plated on a 5% sheep blood agar for counting of ETEC colony forming units at 0, 5, 35, 65 and 125 min after blending. The optical density at 600 nm explained 83% of ETEC colony forming units, indicating that the established linear equation (y= 6E+08x − 4E+07, P<0.004) can be used for robust quantification of ETEC cell density in TSB, sweetener and dextrose media. When ETEC was blended with sweetener and dextrose, survival of ETEC was decreased by 45% and 72% within 5 min post-blending. Therefore, further research is required to find out the suitable medium that has potential to improve palatability without compromising survival of ETEC.

Key words **:** Dextrose, Enterotoxigenic *Escherichia coli*, Survival, Sweetener

I. Introduction

Escherichia coli (*E. coli*) is a facultative anaerobic bacteria with both non-pathogenic and pathogenic strains existing throughout the gastrointestinal tract and feces of pigs (Hampson, 1994; Pluske et al., 2002). Pathogenic *E. coli* associated post-weaning diarrhea (PWD) and edema disease are common diseases in commercially-housed weanling pigs, and it is reported that pathogenic *E. coli* proliferates particularly within 2 weeks after weaning when piglets encounter various socio-psychological and nutritional stressors (Kim et al., 2012; Heo et al., 2013). Studies in relation to PWD are generally conducted in a clean and hygienic research facility and do not reflect the majority of commercial environments, suggesting that results obtained from a research facility may not necessarily represent the conditions of commercial practice. Therefore, an enterotoxigenic *E. coli* (ETEC) oral challenge model has been used to mimic the biological responses observed in commercial conditions, where newly-weaned pigs are exposed to considerable challenges.

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The ETEC serogroup O149; K91; K88 isolated from a clinical case of PWD has been used for experimental infection of young pigs to (sub)clinically express PWD, and trypticase soy broth (TSB) is a commonly used medium for dilution of ETEC after incubation at 37°C (Heo et al., 2010). However, the lower palatability of TSB during oral administration of ETEC during the infection procedure can cause accidental passage of ETEC into the airways, with death occurring within a few hours after infection.

A sweetener (i.e., non-alcohol fruit concentrate) or dextrose were proposed as possible substitutes for TSB to increase palatability. To our knowledge, however, there was no report on survival of ETEC after blending in different media, which has potential to improve acceptability by piglets.

Another problem during the preparation of an ETEC solution for oral infection is that there is no methodology to estimate ETEC cell concentration in the dilution media immediately after dilution. The ETEC concentration has been traditionally determined after overnight incubation at 37℃ following inoculation of the freshly prepared medium on a 5% blood agar plate. As only freshly prepared ETEC solution is used for the oral infection procedure, to mainly prevent possible overgrowth of ETEC, there is a need for quantification of ETEC concentration in the freshly prepared solution.

Therefore, the purposes of this study were to (1) establish a regression equation that can be used for estimation of ETEC concentration in dilution media using colorimetric measurement of cell density; and (2) examine survival of ETEC after blending either in TSB, sweater or dextrose.

II. Materials and Methods

A strain of ETEC, isolated from a clinical case of PWD in Western Australia (serogroup beta-hemolytic *E. coli* O149; K91; F4; toxins LT, STa, STb; Pig Health and Research Unit, Department of Primary Industries, Bendigo, Vic., Australia), was used in this *in vitro* experiment. The ETEC was grown on sheep blood (50 mL/L) agar plates (Columbia base, Oxoid, WA, Australia) (McDonald et al. 2001) and incubated overnight at 37°C. A representative colony was then removed from the plate and seeded into 20 mL of sterile TSB (trypticase soy broth, Becton Dickinson, Franklin Lakes, NJ, USA) in a McCartney bottle and incubated overnight at room temperature (20-25℃). A volume of 4 mL was then aseptically transferred to a larger volume of sterile TSB (400 mL) and incubated at 37℃ for 3-4 h. When the density reached 10^8-10^9 colony-forming units (cfu)/mL, the solution was centrifuged at 3000 × *g* for 15 min at 4℃. The supernatant was discarded and the remaining *E. coli* pellet was suspended either in TSB (100 mL), sweetener (60 mL TSB + 40 mL fruit flavored concentrate; sucrose- and citric-acid based Raspberry Cordial, Cottees, Tullamarine, VIC, Australia), or dextrose (50 mL TSB + 50 mL dextrose; 0.5g/mL dextrose, Hospira, Inc., Lake Forest, IL, USA) to provide similar levels of sweetness.

The ETEC cell density was then measured using a UV-VIS Spectrophotometer (UVmini-1240, SHIMADZU, Kyoto, Japan) at 0, 5, 35, 65 and 125 min after blending in three different media with the absorbance read at 600 nm against blank TSB, sweetener, and dextrose. Also, the characteristic colony morphology of the beta-hemolytic *E. coli* and *E. coli* colony forming unit were examined by plating the solutions onto sheep blood (50 mL/L) agar at 0, 5, 35, 65 and 125 min after blending. Survival of ETEC was expressed as proportion of colony forming units at 5, 35, 65 and 125 min in relation to the colony forming units at time 0 (before blending with the three different media). Linear regression analysis was conducted to establish a relationship between optical density and plated colony forming unit using Genstat 15th edition, VSN International Ltd., Hemel Hempstead, UK).

III. Results and Discussion

The linear relationship between optical density of the ETEC solution at 600 nm (against different media as a blank) and the number of ETEC colony forming units determined after plating the dilution media on 5% blood agar and overnight incubation at 37℃ is presented in Fig. 1. The optical density at 600 nm explained 83% of ETEC colony forming units, and therefore the established linear equation (y= 6E+08x − 4E+07, P〈0.004) can be used for robust quantification of ETEC cell density in TSB, sweetener and dextrose media.

A freshly prepared ETEC solution is recommended for oral ETEC infection as prolonged storage of ETEC in TSB medium generally eventually causes overgrowth of ETEC in the medium and subsequent production of undesirable toxins in the medium. One of the weaknesses of the oral ETEC infection model associated with the use of freshly prepared ETEC solution is that ETEC cell density is unknown when pigs were infected, as it has traditionally been determined by plating the ETEC solution on blood agar plate with overnight incubation at 37ºC. The present *in vitro* study showed that spectrophotometry can be used for estimation of ETEC cell density in infection media before conducting the oral infection procedure. Therefore, quality control of the ETEC infection media can be initiated with the proposed linear regression equation to improve precision of ETEC inoculation density per pig.

Survival of ETEC was measured at 0, 5, 35, 65, and 125 min after blending with the three different media and expressed as a proportion of pre-blending value of colony forming units (Fig. 2). When TSB was used as a blending medium the ETEC concentration was slightly increased to around 110% over the 125 min incubation on ice. However, when sweetener was used as a blending medium, survival of ETEC was immediately decreased to 62% at 5 min post-blending and it was further decreased to 55% at 60 min. When dextrose was used as a blending medium, ETEC survival was sharply decreased to 28% at 5 min post-blending and there was no further reduction over 125 min on ice.

These results indicate that blending of ETEC solution with either sweetener or dextrose significantly decreases ETEC survival and the reduction generally occurs

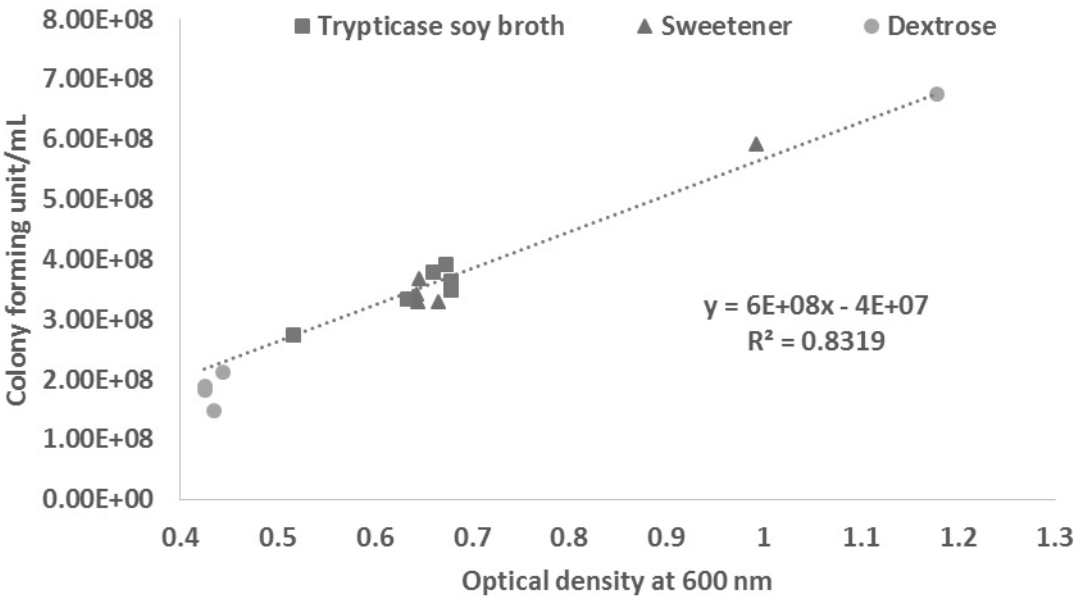

Fig. 1. Relationship between optical density of enterotoxigenic *E. coli* solution at 600 nm (against different media as a blank) and the number of enterotoxigenic *E. coli* colony forming units determined after plating the dilution media on 5% blood agar and overnight incubation at 37ºC.

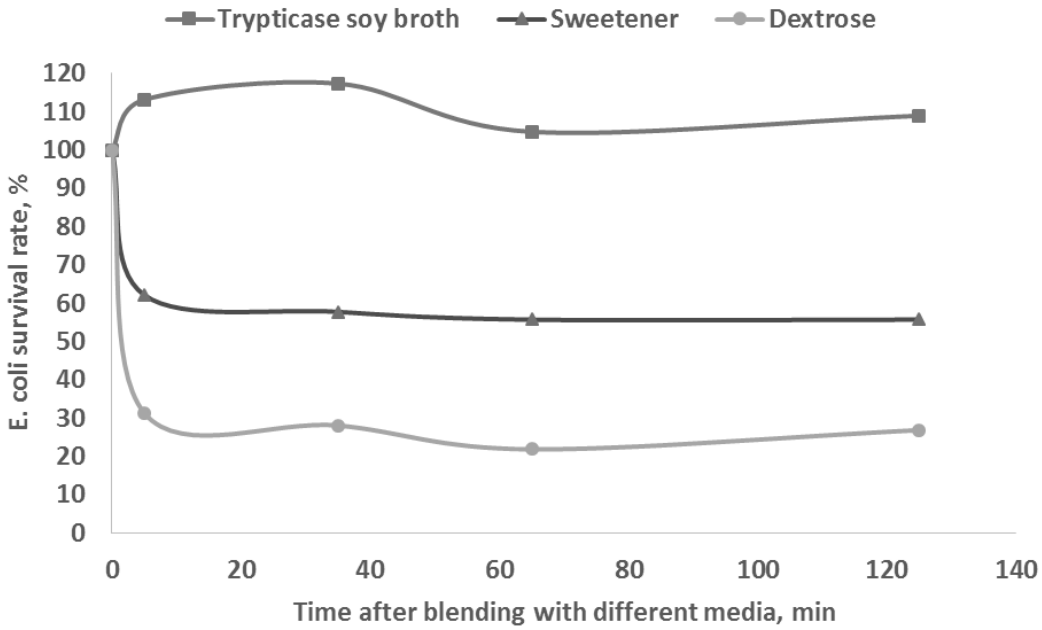

Fig. 2. Effect of dilution media on survival rate of an enterotoxigenic strain of *E. coli* after blending.

within 5 min post-blending. Dextrose (5g/mL) has high osmolality (2,525 mOsm/L), and therefore, blending ETEC solution with dextrose may have increased the osmotic diffusion of dextrose into the *E. coli* cell wall, which resulted in 72% of *E. coli* death within 5 min post-blending (Bianchi and Baneyx, 1999). The sweetener was a fruit-flavored concentrate for human consumption after dilution and contained sucrose and citric acid. Presence of sucrose and citric acid may have increased osmolality and decreased pH of the blended solution (Small et al., 1994), respectively, and resulted in 45% of *E. coli* death within 60 min. Therefore, both sweetener and dextrose are not suitable blending media for the ETEC solution, and further research is required to find out that improves palatability without affecting ETEC survival rate.

IV. Conclusion

The spectrophotometric measure of cell density at 600 nm wave length enabled the successful establishment of a linear regression equation for robust quantification of ETEC cell density in TSB, sweetener and dextrose media. However, blending ETEC solution with either sweetener or dextrose significantly decreased ETEC survival within 5 min post-blending.

Acknowledgements

Authors would like to acknowledge technical support from Ms Josie Mansfield and Aracely Hernandez at Murdoch University. This research financially supported by the research fund of Rural Development Administration, Republic of Korea (Project No. 01011403).

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