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A novel miniaturized most probable number method for the enumeration of *Campylobacter* spp. from poultry-associated matrices

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ABSTRACT

A novel miniaturized most probable number (mMPN) method was developed for the enumeration of thermophilic *Campylobacter* spp. using a modification of blood-free Bolton broth (supplemented with 25 mg/l of sulfamethoxazole) and CampyFood ID agar. The mMPN was evaluated by comparison with direct plating (modified ISO/TS, 10272-2:2006) for the analysis of samples ($n = 149$) representing various poultry matrices (carcases, broiler ceca and feces, scald tank water and feed). A sensitivity of 95%, specificity of 90% and Cohen KAPPA agreement of 0.84 was determined for the mMPN method compared to direct plating. Quantitative comparison found 83% of enumerations to be less than ± 1 $\log_{10}$ different (Student's *t*-test $P<0.001$). Financial analysis showed that the mMPN required 51% less media and 60% less labor than the direct plating protocol. The mMPN provides a method that can be used for complete through-chain analysis that has a single enrichment step and multiple dilutions to extinction for a variety of samples (containing low, medium and high populations).

1. Introduction

Zoonotic *Campylobacter* spp. are the leading cause of human foodborne bacterial gastroenteritis worldwide (FAO/WHO, 2009). The handling and consumption of raw or undercooked poultry meat are regarded as a significant risk factor for human campylobacteriosis (Eberhart-Phillips et al., 1997; Friedman et al., 2004; Kapperud et al., 1992; Neimann et al., 2003; Stafford et al., 2007). Both *C. jejuni* and *C. coli* colonize the intestinal tracts of avian species, proliferate to high populations during rearing, and are widely disseminated during slaughter (Beery et al., 1988; Berrang et al., 2000; Corry and Atabay, 2001; Stern et al., 2001). Therefore, measures for the prevention and control of enteropathogens, including *Campylobacter*, are applied throughout the poultry production chain to raw materials, environmental sources and finished raw product (Cox and Pavic, 2010).

Methods to assess the microbiological risk (exposure) to humans and the efficacy of associated poultry management strategies need to be reliable and accurate (Hoornstra and Notermans, 2001). Consequently, numerous qualitative detection methods have been developed; the ISO 10272-1:2006 horizontal method (International Organization for Standardization, 2006a) is commonly used. However, due to the high isolation rate of campylobacters from poultry-associated samples, numerical estimations are more meaningful. While quantitative data can be achieved by direct plating on selective agars (ISO/TS, 10272-2:2006 and AS 5013.6-2004), enumeration of *Campylobacter* spp. in matrices containing large populations of background microflora, can be difficult and may require several (dilutions and replicate) plates to obtain a count.

Historically, many detection and enumeration methods for microorganisms have been developed but with varying degrees of success in application (as reviewed by Cox and Fleet, 2003). The most probable number (MPN) method was first published by McCrady (1915) and exploits Poisson's distribution for the enumeration of (target) organisms per unit of analyte. The MPN method combines the sensitivity of growth in an enrichment broth with multiple tubes to obtain a quantitative data set. Studied extensively and widely accepted, this method is adopted when other more accurate and precise methods of enumeration are impractical (Bolton et al., 1982; Line et al., 2001; Russell, 2000). However, the standard MPN method is very labor intensive and time-consuming.

Miniaturization of microbiological assays by means of reducing culture media volumes has been an innovative step in recent decades (Fung, 1992). Miniaturized MPN methods, which can be performed quickly, accurately and cheaply, without affecting sensitivity or specificity, have been developed to facilitate enumeration of several target organisms (Jagals et al., 2000; Line and Siragusa, 2006; Pavic et al., 2010). While these authors all described success in miniaturization, there are no reports of a miniaturized MPN method that uses the ISO-prescribed Bolton broth for the enumeration of *Campylobacter* spp. from poultry matrices, which include high background microflora (ceca and feces) and environments harsh to the target bacterium (scald tank water, feed and broiler carcases).

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This paper describes the development and comparison, to direct plating, of a quantitative mMPN method for the enumeration of *Campylobacter* spp. from critical poultry-associated reservoirs. The mMPN design was based on a procedure developed by Pavic et al. (2010) for the enumeration of *Salmonella* from poultry matrices, but incorporated a modification of blood-free Bolton broth and a Campylobacter selective plating medium.

2. Materials and methods

2.1. Preparation of inocula

The *C. jejuni* and *C. coli* strains used in this study were reference cultures and field isolates. Culti-Loops of *C. jejuni* ATCC 29428 (Oxoid, CL1410, Hampshire, UK) and *C. coli* ATCC 33559 (Oxoid, CL9039) were stored at −20 °C. The other three strains (*C. jejuni* JC 841, JC 218; *C. coli* JC401) were from an Australian commercial chicken meat (broiler) producer. These strains were isolated using an ISO/TS 10272-2:2006 equivalent method and preserved at −80 °C in FBP medium (Gorman and Adley, 2004).

All isolates were grown in 100 ml of blood-free Bolton broth (BFBB; Oxoid, CM0983 and SR0183) at 37 °C for 24 h under microaerobic conditions using GENbox microaer sachets (bioMérieux, ref 96125, Marcy-l'Etoile, France) in GENbox jars. Culture purity was verified on Sheep Blood Agar (SBA; bioMérieux, ref 04319) and confirmed using BAX® real-time PCR (Oxoid, QB3449), according to the manufacturer's instructions.

2.2. Detection and enumeration of Campylobacter

2.2.1. Direct plating

All samples were tested at a laboratory accredited by the National Association of Testing Authorities (Australia) using, as a quantitative reference method, ISO/TS 10272-2:2006 with the following internally validated modifications: a 0.1 ml aliquot of sample or appropriate dilution was plated directly on CampyFood ID agar (CFA; bioMérieux, ref 43471) and *Campylobacter* Skirrow Agar (CSK; bioMérieux ref 04025). Suspect colonies (flat gray on CSK, entire burgundy red on CFA) were then confirmed using BAX® real-time PCR.

2.2.2. Qualitative method

Samples were enriched in BFBB at 42 °C for 48 h under microaerobic conditions. A 10 µl aliquot of enriched sample was streaked on CFA and CSK, with plates incubated at 42 °C for 48 h. Typical colonies were selected and confirmed using BAX® real-time PCR.

2.2.3. Miniaturized most probable number (mMPN)

A 1 ml volume of the suspension, as described in Sections 2.3 and 2.4, was pipetted in triplicate into each of three empty plasma tube wells (i.e. A1 to A3) (Scientific Specialties Inc, 1720-00, Lodi, CA, USA). Serial decimal dilutions (100:900 µl) of the original suspension were performed using a multi-channel pipette in racked plasma tubes (Scientific Specialties Inc, 1750-00) in single-strength modified BFBB (mBFBB; containing 25 mg/l sulfamethoxazole [SMX, Sigma-Aldrich S7507, Castle Hill, Australia] dissolved in dimethylsulfoxide [DMSO; Sigma-Aldrich 472301]) to generate the 3-tube MPN (Fig. 1). All tubes were mixed with repeated aspiration. The triplicate dilutions were incubated for 24–48 h at 42 °C under microaerobic conditions.

From each of the dilutions in plasma tubes post-incubation, 50 µl aliquots were transferred into a V-bottomed microtiter plate (Greiner bio-one, 651101, Gloucestershire, UK) with each dilution in a subsequent row (e.g. 10^{-1} in A1 to A3, 10^{-2} in B1 to B3, to a theoretical maximum dilution of 10^{-8} in row H1 to H3). The V-bottomed microtiter plates were pre-filled with 100 µl per well of complete BFBB + 25 mg/l SMX with 0.3% bacteriological agar (Oxoid Agar No. 1 LP0011) and 150 µg/ml 2,3,5-triphenyltetrazolium solution (TTC; Sigma-Aldrich T8877). The plate was then covered with an adhesive plastic film (Seal Plate®; Excel Scientific Inc, Victorville, CA, USA) and incubated (42 °C/24 h, microaerobic).

Red color development in a well was considered a presumptive positive result for the presence of *Campylobacter* spp. A 3 × 3 tube MPN was confirmed by subculturing on CFA and isolates were speciated using BAX® PCR. When more than three dilutions had red-colored wells, the most diluted set yielding all suspect positive wells, followed by the two successive dilutions, was selected for streaking. The combination of positive and negative wells yielded a MPN data set.

2.3. Comparison using pure cultures

The performance of the mMPN and reference methods was assessed using the five *C. jejuni* and *C. coli* strains. The inocula were prepared as described in Section 2.1 and decimally diluted (in triplicate), according to the testing procedure, to the target concentrations of 10^2, 10^4 and 10^6 cfu/ml ($n = 45$).

2.4. Comparison using naturally and artificially contaminated poultry matrices

The comparison of the two quantitative methods was performed using matrices containing varying levels of both background microflora and target organism. The matrices were broiler feces, broiler

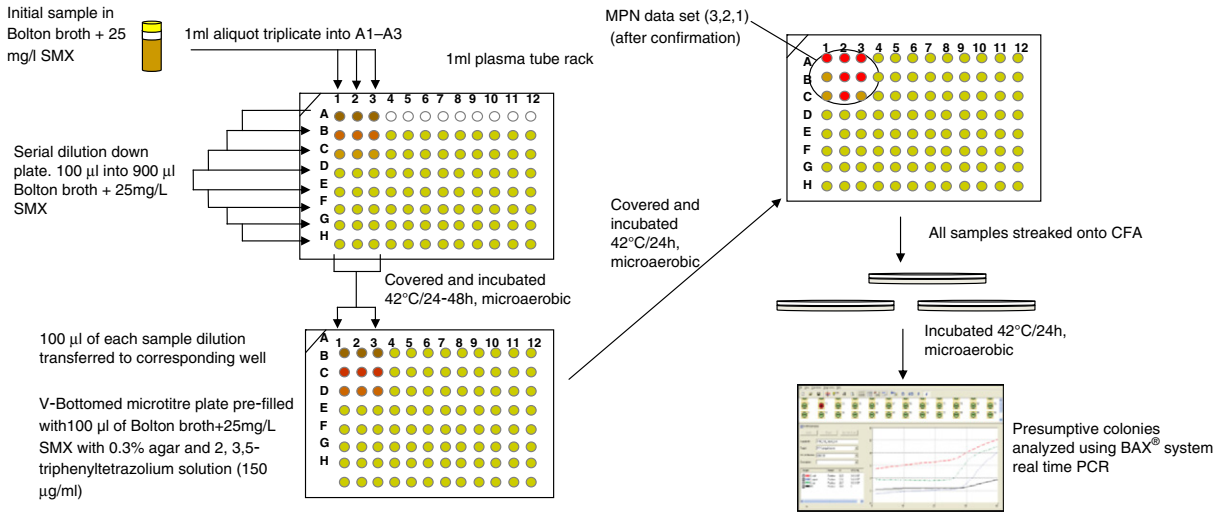

Fig. 1. Schematic of the miniaturized Most Probable Number method for the enumeration of *Campylobacter* spp.