Peer reviewed **ORIGINAL ARTICLE**

EFFICACY OF PHENOTYPIC, PCR AND MALDI-TOF IDENTIFICATION METHODS FOR *CAMPYLOBACTER SPP.*

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ABSTRACT

This study compared phenotypic and genotypic identification methods of *Campylobacter spp.* against the polymerase chain reaction (PCR) in terms of sensitivity, specificity, positive-predictive value and negative-predictive value. Thermophilic *Campylobacter* isolates were identified using conventional biochemical tests, specifically hippurate hydrolysis, matrix assisted laser desorption ionization- time of flight (MALDI-ToF) mass spectrometry and PCR with primers unique to *C. jejuni* and *C. coli*. MALDI-ToF was shown to be superior to biochemical tests for identification of *C. coli* but equivalent to biochemical tests for *C. jejuni*.

KEYWORDS

Campylobacter, PCR, MALDI-ToF

INTRODUCTION

Most routine laboratories lack discriminatory tests for species differentiation of *Campylobacter* bacteria.[1] Biochemical tests were the initial form of bacterial identification and are based on the metabolic characteristics of bacterial species. Together with a dichotomous key tree, the results of a series of biochemical tests can distinguish between bacteria, even if they are closely related. Such tests are also used to identify *Campylobacter*.[2] PCR identification for *Campylobacter* remains the popular tool despite the methodology being time-consuming and labour-intensive. Standardisation difficulties due to very different primers and PCR target genes having been published to optimise PCR identification for *Campylobacter*.[3] The MALDI-ToF mass spectrometry apparatus has the ability to identify pure bacteria culture within a relatively short time (less than an hour), and without any biochemical testing. It is less labour-intensive, particularly when opting to use the direct method (indirect method also available), and much less sample preparation is needed. In principle, MALDI-ToF mass spectrometry uses a 'soft' ionization technique by irradiating the crystalline structure of a prepared sample, resulting in the production of gas-phase ions. The ions accelerate through an electric field that is detected and measured by calculating the 'time-of-flight' (TOF), with smaller molecules travelling faster and larger molecules take longer, resulting in a spectrum compiled from the masses measured. The spectrum created is compared to an existing data base, with the relatedness being produced as a numeric score.[4,5] This study investigated the MALDI-ToF as a diagnostic alternative.

MATERIALS AND METHODS

Campylobacter *strains and pre-screen identifications*

Sixty nine isolates forming part of a larger collection of isolates collected from poultry as previously published[6] constituted the study sample. These isolates were screened as thermophilic *Campylobacter* using conventional methods. These included, Gram staining, observation of the unique spiral morphology, growth at 41.5°C, naladixic acid and cephalothin disc susceptibility, oxidase test and hippurate hydrolysis. *C. jejuni* spp. jejuni was positive and *C. coli* was negative for the hippurate hydrolysis tests.

PCR

PCR identification of *Campylobacter* was conducted according to the methods outlined by Linton *et al.*,[7] and DNA extraction was accomplished using the heat-lysis method. DNA samples were quantified spectrophotometrically using the NanoDrop 2000 (ThermoScientific, South Africa), and standardised to 15 ng/μl with nuclease-free water. To identify *C. jejuni*, a 735 base pair region of the hippurate gene (HipO) was amplified using the following primers: forward 5´-GAA GAG GGT TTG GGT GGT-3´ and reverse 5´-AGC TAG CTT CGC ATA ATA ACT TG-3´.[7] For *C. coli* identification, a 500 base pair region of the putative aspartokinase gene was amplified using the primers: forward 5´-GGT ATG ATT TCT ACA AAG CGA G-3´ and reverse 5´-ATA AAA GAC TAT CGT CGC GTG-3´.[7]

MALDI-ToF analysis

Isolates were identified using MALDI-ToF mass spectrometry (Autoflex, Bruker Daltonics) by the direct application method. A small quantity of 24-48 hr culture colonies from a micro-aerophilic atmosphere were smeared onto a microtitre spot of a 384 polished steel plate (Bruker Daltonics, Bremen, Germany). This was overlaid with 1μL matrix, which is a saturated cyano-4-hydroxycinnamic acid solution (HCCA) (Bruker Daltonics). Calibration was done with the Bruker Daltonics bacteria test standard (BTS), which is an *E. coli* control sample. Each isolate was performed in duplicate and set in linear mode with the parameters fixed at mass range of 2-20 kDa, ISI 20 kV, ISI 18.62 kV, lens 6.5 kV. Spectra were analysed using the MALDI Bioty-

per 3.0 software (Bruker Daltonics), and the Bruker Genotypic database was selected for the identification framework. The peaks generated were automatically compared to the data in the genotypic data base and its probability conveyed to a score. The range of scores and descriptions were analysed as follows: 2.300 to 3.000 were highly probable species identification; 2.000 to 2.299 were a secure genus identification and probable species identification; 1.700 to 1.999 were probable genus identification; and 0.000 to 1.699 were not a reliable identification (Bruker Daltonik MALDI Biotyper classification results).

RESULTS

Identification and specificity

The sensitivity, specificity, positive and negative predictive values of the hippurate and MALDI-ToF mass spectrometry were compared with PCR methods as the gold standard to identify *C. coli* and *C. jejuni* bacteria (See Table 1). Of the 69 isolates, 26 (38%) were identified as *C. jejuni* and 38 (55%) as *C. coli*, with a single isolate identified as *C. lari*. The MALDI-ToF mass spectrometry was shown to be superior to the biochemical tests to identify *C. coli*, but equivalent to the biochemical tests for *C. jejuni*.

DISCUSSION

Although a series of biochemical tests can distinguish between bacteria, even if they are closely related, a percentage of natural 'exception to the rule' may exist,[8] and together with technical errors in the laboratory, identifying a bacterial species can be incorrectly concluded. The hippurate hydrolysis test is traditionally used to distinguish between *C. jejuni* and *C. coli*, but is subject to inaccuracy if the laboratory execution was not optimal. In addition, the existence of hippurate-negative *C. jejuni* species is well-documented[9-11] and could consequently be incorrectly defined as a *C. coli* species. This was verified with a single isolate from the rural farming system that was identified as a hippurate-negative *C. jejuni*, but without PCR would have been incorrectly identified as a *C. coli* isolate. PCR identification for *Campylobacter* remains the gold standard as PCR primers chosen were very specific for either *C. jejuni* or *C. coli*, and excluded other species. This was highlighted when testing isolate CA 160, with results showing that it was neither *C. jejuni* nor *C. coli*. This isolate was found to be 99% related to the *C. lari* ATCC 35221 strain when using primers that target the 16S ribosomal RNA gene.

Bessède *et al.* (2011) compared the identification accuracy of MALDI-ToF with PCR methods. It was shown to be 100% comparable with most thermophylic *Campylobacter*s, and indicated a 0.6% error on *C. jejuni*.[12] Here, the indirect method has shown to be 100% accurate and could be used as an alternative.

The indirect method is an extraction technique where the cell suspension is pre-treated with ethanol followed by formic acid and acetonitrile treatment to disrupt the cell walls, after which the supernatant is dispensed on the polished steel spot. Alispahic *et al.*, (2010)[13] were able to achieve a 100% accuracy using the indirect method. Dendogram peak analysis was also accurately achieved when compared to PCR analysis using main spectra projection (MSP) and principle component analysis (PCA) software.[14] However, the addition of the software can become labour-intensive, where the MALDI-ToF is largely reserved as a research tool as opposed to a routine diagnostic tool, with many studies looking at large scale diagnostic laboratory application.

If the MALDI-ToF mass spectrometry is going to replace traditional PCR methods as an identification tool, it might be worth the Bruker Daltonik agency continually updating their Bruker Genotypic reference data base, as new *Campylobacter* species and strains are frequently documented. It is thus concluded that identifying *Campylobacter* species is challenging, given that current PCR methods are not standardised. However, MALDI-ToF spectrometry is a possible alternative diagnostic tool.

FUNDING

This work was supported by the National Research Foundation Thuthuka: Researchers in Training Programme Ref. TTK2007040500009.

TRANSPARENCY DECLARATIONS

None to declare.

ACKNOWLEDGEMENTS

The authors would like to sincerely thank the 'Catalysis and Peptide Research Unit' at the University of KwaZulu-Natal for the training and use of their MALDI-ToF spectrometer. Special thanks to Ms Marian Bezuidenhout for training on the MALDI-ToF MS.

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Table 1: Comparison of hippurate and MALDI tests to the gold standard (PCR) for sensitivity, specificity, positive predictive and negative predictive values

Test	Sensitivity	Specificity	Positive predictive	Negative predictive
Hippurate (*C. coli*)	100 %	86.7 %	90.5 %	100 %
Hippurate (*C. jejuni*)	96.3 %	100 %	100 %	97.6 %
MALDI-ToF (*C. coli*)	100 %	96.7 %	97.4 %	100 %
MALDI-ToF (*C. jejuni*)	96.3 %	100 %	100 %	97.6 %

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