CAMPYLOBACTER DETECTION IN FOOD USING AN ELISA-BASED METHOD

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Abstract

As one of the most important cause of acute bacterial gastro-enteritis in humans, *Campylobacter jejuni* and *Campylobacter coli* need to be quickly and easily monitored in the food chain. A new method, called Transia Plate *Campylobacter*, combining a two-step enrichment done in Bolton broth and an ELISA test, has been developed for the food application.

The ELISA test detected all the different *Campylobacter* strains (13 *Campylobacter jejuni*, 17 *Campylobacter coli*, 2 *Campylobacter upsaliensis* and 3 *Campylobacter fetus*), and did not show any cross-reactivity towards the other bacteria belonging to different genera (n=46).
The limit of detection of the ELISA test, evaluated with 4 *Campylobacter jejuni* and 4 *Campylobacter coli* strains, was found between 10^5 and 10^6 CFU/ml.

The studies of spiked food samples (raw milk, fish filet, raw milk cheese, rapped cabbages, pork filet and poultry) contaminated by different levels of *Campylobacter jejuni* or *Campylobacter coli*, showed that the limit of detection of the method was lower than 10 CFU/25 g of food. Finally, when screening naturally contaminated food samples (239 samples, such as raw and processed meat products (n=95), dairy products (n=99), seafood products (n=30) and vegetables (n=15), the Transia Plate *Campylobacter* method offered equivalent results to the ISO method 10 272/1995.

Introduction

Among the food-borne pathogens, *Campylobacter* genus is responsible for the highest number of cases of human enteritis in the US, UK, Belgium, Sweden (Federighi et al., 1997 ; Friedman et al., 2000) and also in developing countries (Oberhelman and Taylor, 2000). Three *Campylobacter* species, *C. jejuni*, *C. coli* and *C. lari*, account for more than 99% of the human isolates, of which *C. jejuni* represents about 90% (Hunt *et al.* 1997).

Food, especially raw poultry products, unpasteurised milk and water are the major vehicles in the transmission of *Campylobacte*r to humans. To prevent human contamination, many countries require routine food testing.Using conventional bacteriological methods (ISO 10272 / 1995, FDA BAM 8th edition / 1998, USDA / FSIS Microbiology Laboratory Guidebook, 3rd edition / 1998) for detecting *Campylobacter* in food is often a challenge:

- The isolation from food samples with a high background flora is difficult.
- Detection requires different specific media and the performance of these media can vary considerably depending on the food type.
- *Campylobacter* grows slowly under microaerobic atmosphere and it takes many days before isolation can be achieved.

The trend is to use alternative methods, such as antibody or DNA based assays which offer a quicker, simpler and more robust solution. Nevertheless, those methods still require cultural steps.

Transia Plate *Campylobacter* is a new ELISA test dedicated to food analyses using antibodies specific for *Campylobacter*. This immunoassay is carried out after a 2-step-enrichment protocol (totally 44 to 48 hours) using Bolton media incubated at 42°C.

The main goal of this work was to determine the performance of this Transia Plate *Campylobacter* method in terms of specificity, sensitivity and limit of detection with pure strains, but also with naturally and artificially contaminated food samples and in comparison to the ISO method.

Material and Method (1)

➢Material

Bolton broth and its supplement were purchased from IDG Limited (Bury, UK). Casein soya broth, Yeast extract and Columbia agar were supplied by Biokar (Beauvais, France). Modified Charcoal Cefoperazone Desoxycholate agar (mCCDA) and its supplement, Karmali agar and its supplement, horse and sheep blood, the Nutrient broth No 2 used for the preparation of the Preston broth and its supplement, the Maximum Recovery Medium (MRD), the nalidixic acid and cephalotin discs and the Campygen were supplied by Oxoid (Basingstoke, UK). The reagents for the oxydase, sodium hippurate and indoxyl acetate tests were purchased from Sigma (St Louis, USA). The Transia Plate *Campylobacter* kit was manufactured by Diffchamb.

➢Reference method (NF-EN ISO 10272/95)

Twenty-five grams of sample were mixed with 225 ml of Preston broth in a stomacher bag and incubated for 18 hours at 42°C. At the end of this enrichment step, streakings were done onto both mCCDA and Karmali agar plates, which were then incubated for 2-5 days at 42°C. All the incubations were done in a jar in a microaerobic atmosphere.

➢Transia Plate Campylobacter Method

The enrichment protocol is presented on figure 1.

The ELISA procedure is presented in figure 2.

➢Result Interpretation

The positive threshold (PT) was calculated by adding 0.10 to the optical density (OD) of both negative controls (NC).

PT = [(NC1 + NC2) / 2] + 0.10

Any sample with an OD higher than or equal to PT is considered positive for Campylobacter.
The negative threshold (NT) was calculated as follows : NT = PT x 0.9
Any sample with a OD lower than NT is considered negative for Campylobacter. Any sample with its OD between PT and NT is considered doubtful. Both positive and doubtful results must be confirmed by further identification.

➢Confirmation of the results

Each enriched sample was streaked onto mCCDA agar and incubated for 48 hours at 42°C under microaerobic atmosphere (μO_2). A gram coloration was performed with one to ten suspected *Campylobacter* colonies. If typical *Campylobacter* bacilli were observed, they were identified by checking the presence of a catalase and an oxydase, then by studying their growth onto sheep blood agar, under different incubation conditions (37°C μO_2, 25°C μO_2, 42°C μO_2 and 37°C in aerobic condition). Finally, traditional confirmation tests (hippurate hydrolysis, indoxyl acetate hydrolysis, susceptibility to nalidixic acid and cephalotin (30 µg disc)) were used to complete the identification.

Figure 1. Sample Preparation Flow Chart for the Transia Plate Campylobacter Tests

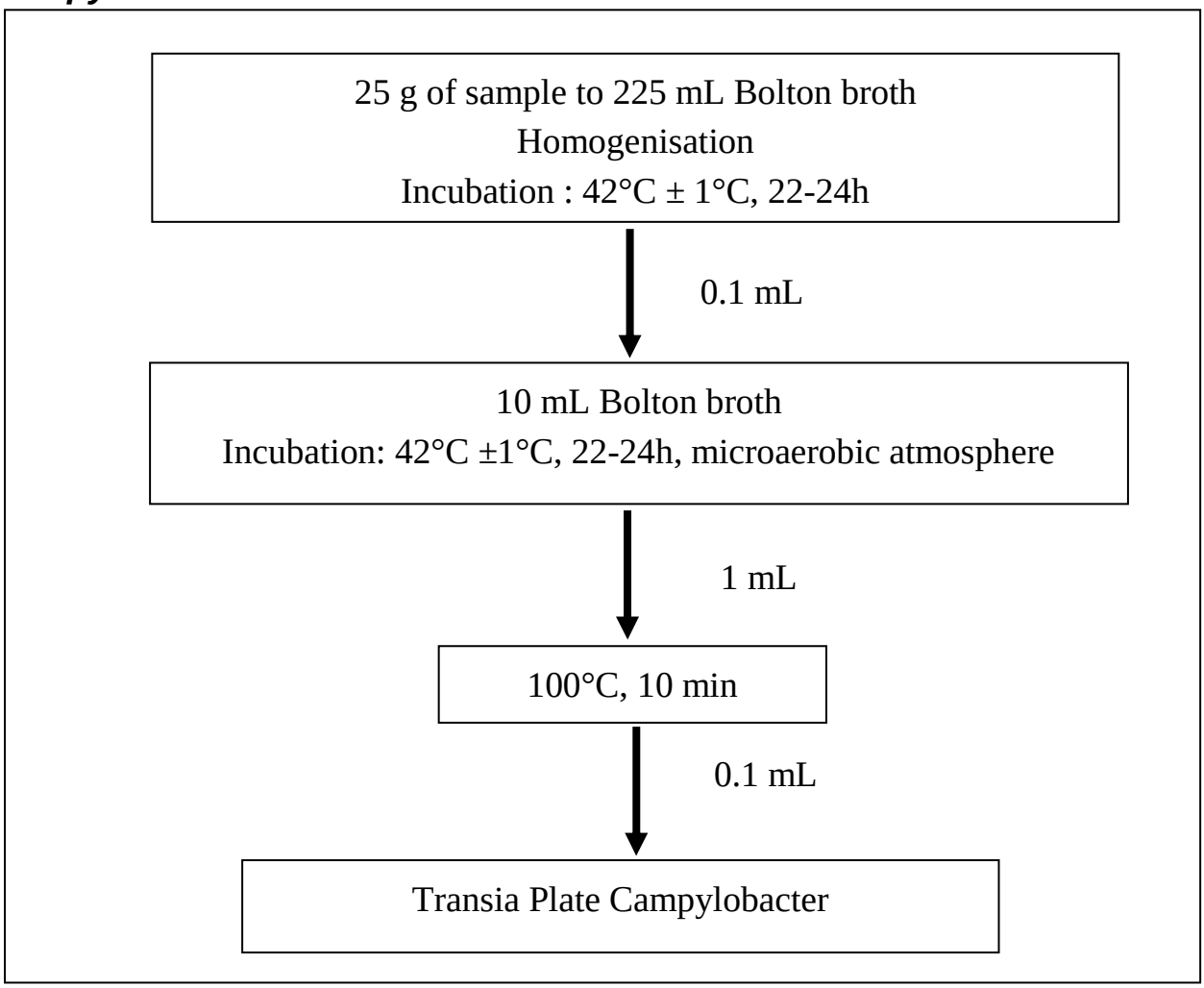

Figure 2. ELISA procedure

This immunoassay uses breakable 8-well-stips coated with antibodies directed against specific Campylobacter antigens. All the reagents are supplied in a ready to use format.

Step 1

Distribute the controls (negative and positive) and the samples into the wells. Incubate for 45 minutes at 37°C. Then wash each well 5 times.

Step 2

Distribute the conjugate (anti-Campylobacter antibodies labelled with peroxydase enzyme) into each well.

Incubate 10 minutes at room temperature.

Step 3

Distribute the substrate/chromogen mixture to each well.

Incubate 10 minutes at room temperature.

Step 4

Add the stop solution to each well. Read the optical densities at double wavelength 450/620 nm.

Material and Method (2)

➢Specificity Study

The specificity of the ELISA was evaluated with 35 *Campylobacter* strains and 46 non-*Campylobacter* strains. For the *Campylobacter* strains, colonies were inoculated into Bolton broth without supplement and incubated for 24 hours at 42°C. After incubation 0.1 ml was transferred into 10 ml of Bolton broth with supplement, and incubated from 48 hours to six days at 42°C. Both incubations take place in microaerobic atmosphere.

The non-*Campylobacter* strains were tested after a culture in TS/YE broth for 24 hours at 37°C, and also after a subculture of 0.1 ml into 10 ml of Bolton broth without supplement, incubated for 24 hours at 42°C (or at their optimal growth temperature).

➢Detection Limit of the Immunoassay

Four *Campylobacter jejuni* and four *Campylobacter coli* strains were analysed. After a pre-culture in Bolton broth without supplement, 24 hours at 42°C, the strains were inoculated in Bolton broth with supplement (0.1 ml + 10 ml) and incubated for 48 hours at 42°C. Both incubations were done in a microaerobic atmosphere. Several dilutions were then, performed in supplemented Bolton broth and tested by ELISA after boiling. Accurate enumeration of the bacteria was done in parallel by dispatching ten spots of 10 µl for two or three dilutions of the bacterial culture (10^{-4}, 10^{-5} and 10^{-6}) onto sheep blood agar plates.

➢Study of artificially-contaminated food samples

Six food matrix/*Campylobacter* strain couples were studied: raw milk/*Campylobacter jejuni*-1543, fish fillet/*Campylobacter jejuni*-1544, raw milk cheese/*Campylobacter coli*-1545, poultry/*Campylobacter coli*-1542, pork/*Campylobacter coli*-1545 and coleslaw/*Campylobacter jejuni*-1542.

In order to, as far as possible, test the same samples with the same competition flora, about 700g of each food matrix were homogenised in a Waring blender, then divided into 25g portions. The preparation of the samples was always done the day before contamination, the samples being stored at 2-8°C until the beginning of the experiment. Each food matrix was contaminated at three theoretical inoculum levels: 3, 20 and 100 cells/25 g. The spiking was done in a stomacher bag after blending the sample into the enrichment broth. The concentration of the contaminating suspension was estimated by accurate enumeration onto sheep blood agar.

Non-contaminated samples were tested in parallel according to the same enrichment protocol. The experiment was repeated three times for each level and each method,. The reference method was performed simultaneously.

➢Naturally-contaminated food samples

Two hundred and forty samples (95 meat and 30 fish products, 100 dairy products and 15 vegetables) were purchased at various supermarkets and analysed with Transia Plate *Campylobacter* and the reference methods. It was not possible to find naturally-contaminated dairy and seafood products, and artificial contamination with 50 to 100 *Campylobacter* cells/25g of product was therefore done for 34 samples.

All the samples were divided into two parts after homogenisation in a Waring blender, before being tested.

Results and Discussion

➢Specificity and Detection Limit of the Immunoassay

The overall *Campylobacter* strains (n= 35) were specifically detected by the immunoassay (Table I) while non-*Campylobacter* strains produced negative results (Table II). That confirms the high specificity of the Transia Plate *Campylobacter* assay for the main *Campylobacter* species.

For the 8 *Campylobacter* strains, the immunoassay showed a quite homogeneous limit of detection, from 3.10^5 to 3.10^6 CFU/ml.

➢Limit of detection with artificially contaminated samples

According to the food matrix, the total viable count, before spiking, varied between 4.102 and 3.108 CFU/g (Table III). Transia Plate Campylobacter recovered a higher number of contaminated samples than the reference method (Table IV). The positive ELISA results were all confirmed after streaking, whatever the levels of contamination. Four low-contaminated samples (one fish, two cheeses and one chicken scallop) were found negative by ELISA and were confirmed negative after streaking: the negative results could most probably be explained by the lack of contaminating bacteria.

While the contamination levels of Campylobacter were low (1- 7 CFU/25g) compared with the background (1 to 7 log higher), the Transia Plate Campylobacter method recovered most of the samples. These results confirm that the enrichment protocol was effective for the specific growth of Campylobacter in the presence of a high background flora.

For the different food matrices, the Transia Plate Campylobacter method confirmed a detection limit lower than 10 cfu/25g of product, regardless of the competition flora.

The cultural method (ISO 10 272/95) missed various spiked samples, well spread among the 6 food types. The weaker performance of the ISO method may be explained by the difficulties encountered reading the agar plate, or/and growing Campylobacter on the agar plates, or/and by the better performances of the Bolton broth in growing Campylobacter cells.

Table I: Specificity Results with Campylobacter strains.

Strains	Number of strains	TP *Campylobacter* Positive Results
C. jejuni	**13**	**13**
C. coli	**17**	**17**
C. upsaliensis	**2**	**2**
C. fetus	**3**	**3**

Table II: Specificity results with different bacterial genera.

Strains	Number of strains	TP *Campylobacter* Positive Results
Citrobacter	**3**	**0**
Enterobacter	**6**	**0**
Hafnia	**3**	**0**
Klebsiella	**3**	**0**
Kutrhia	**1**	**0**
Listeria	**2**	**0**
Bacillus cereus	**1**	**0**
Streptococcus	**3**	**0**
Staphylococcus	**4**	**0**
Brochotrix	**1**	**0**
Salmonella	**4**	**0**
Escherichia	**3**	**0**
Propionibacterium	**1**	**0**
Pseudomonas	**2**	**0**
Erysipelothrix	**1**	**0**
Proteus	**2**	**0**
Jonesia	**1**	**0**
Yersinia	**1**	**0**
Shigella, Serratia	**3**	**0**
Ranhella	**1**	**0**

➢Naturally contaminated food samples

In total, both methods showed 48 common positive samples (Table V), the ISO finding six supplementary positive results and the Transia Plate *Campylobacter* method eight supplementary positive results, all of which were confirmed after streaking onto CCDA agar plates. Most of the discrepancies were obtained with the dairy products (respectively four and three supplementary positive samples for ISO and Transia Plate Campylobacter methods), and the raw and processed meat products (respectively two and three supplementary positive samples for ISO and Transia Plate *Campylobacter* methods).

The global percentage of agreement between both methods was 94%. The pair-wise statistical analysis of these 240 samples using the Mac Nemar method concluded that both methods were not statistically different (χ^2=0.28<3.85).

The discrepancies observed between the two methods could be explained partly by:

- Non-homogeneous contamination of the samples by *Campylobacter* as both methods did not use the same broth for the first enrichment step. This means that one sample fraction was contaminated but not the other.
- Different enrichment protocols were used in the two methods, which could stimulate the growth of the *Campylobacter* in different ways, depending on the competition flora, the food matrix, the level of cellular stress, etc.

Table III: Total Viable Count in the food matrices before spiking.

Food Matrix	**Level of the background flora before spiking.**
Fish filet	**2.10^6 CFU/g**
Raw milk	**5.10^3 CFU/g**
Raw milk cheese	**3.10^7 CFU/g**
Coleslaw	**4.10^2 CFU/g**
Pork filet	**4.10^6 CFU/g**
Chicken scallop	**3.10^8 CFU/g**

Table IV: Results for the artificially contaminated samples.

		Transia Plate *Campylobacter*		ISO 10272
Levels of Contamin-ation	***Number of samples***	**Positive ELISA**	**Confirmation**	**Positive results**
0	18	0	0	0
1-7	*21*	17[a]	17[a]	12[b]
13-47	*18*	18	18	16[c]
63-235	*15*	15	15	15
Total Positive samples	***54***	**50**	**50**	**43**

a : negative samples = 1 fish, 2 cheeses, 1 poultry. TPC negative, confirmation negative.
b : negative samples = 1 fish, 3 cheeses, 2 vegetables, 2 pork filets, 1 poultry.
c : negative samples = 1 cheese, 1 vegetable.

Table V: Results of the overall naturally contaminated food samples.

ISO Method	**Positive**	**Negative**	**Total**
TP *Campylobacter*			
Positive	**48**	**8**	**56**
Negative	**6**	**178**	**184**
Total	**54**	**186**	**240**

Conclusions

The Transia Plate *Campylobacter* method has proved to be as sensitive and specific as the ISO reference method for detecting *Campylobacter* in food. The negative and presumptive positive samples are identified within just two days, which reduces the laboratory time considerably (usually four to six days) and decreases the delays in obtaining the results. This method can be applied to different kinds of food matrices, such as dairy products, raw and processed meat, fish and vegetables, regardless of the competition flora.

The two-step enrichment protocol requires a unique broth (Bolton broth), and only the second step is carried out under microaerobic conditions. This innovative enrichment procedure allows *Campylobacter* detection by ELISA after 44 hours incubation.

Because of the microplate format, the assay may be performed manually or by using the fully-automated immunoanalyser, Transia Elisamatic II. The complete Transia Plate *Campylobacter* method can be implemented easily at any food analysis laboratory and contributes to the optimisation of the workflow.

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