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Identification of unusual *Campylobacter*-like isolates from poultry products as *Helicobacter pullorum*

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H.I. ATABAY, J.E.L. CORRY AND S.L.W. ON. 1998. Twenty-six unclassified *Campylobacter*-like strains previously isolated from 15 chicken carcasses and caecal contents, together with two more strains isolated from chicken faeces on a different occasion, were identified as *Helicobacter pullorum* using various phenotypic identification methods. API Campy identification kits and a 16-test identification scheme developed for campylobacters failed to identify these bacteria, or identified them as *Campylobacter* spp. Eighteen strains (including the two isolated on a different occasion) were chosen for examination using a more comprehensive probabilistic identification scheme. Using this method, 14 of the 18 strains were identified as *H. pullorum* with ID scores $>95\%$; two strains were also identified as *H. pullorum* with lower ID scores. Of the remaining two strains, one was not identified with this scheme and the other was misidentified to the *H. acinonyx pylori* complex. Whole cell protein profiling by SDS-PAGE confirmed the identity of these isolates as *H. pullorum*, affirming the value of a polyphasic approach for accurately identifying campylobacteria. The comparatively high prevalence of *H. pullorum* in poultry determined in this study (60%) suggests that routine isolation and identification methods should be amended to enable a thorough evaluation of its role in human gastroenteritis and avian hepatitis. Some phenotypic characters useful in identifying poultry campylobacteria are presented which could be utilized, along with other technique(s), for improved differentiation of the campylobacteria that are found in poultry.

INTRODUCTION

In order to encompass the genera *Campylobacter*, *Arcobacter*, *Helicobacter* and *Anaerobiospirillum* the term 'campylobacteria' has been used (On and Holmes 1995). Campylobacteriosis is recognized as the most common form of acute bacterial gastrointestinal infection in many developed countries (Pearson and Healing 1992; Tauxe 1992; ACMSF 1993). The majority of infections are attributed to the so-called 'thermophilic campylobacters' (*Campylobacter jejuni* subsp. *jejuni*, *Camp. coli* and *Camp. lari*) and poultry meat is considered an important source (Tauxe 1992). However, it is becoming increasingly clear that other species of campylobacteria other than the thermophilic campylobacters may also be human pathogens (Fennel *et al.* 1986; Edmonds *et al.* 1987; Tee *et al.* 1988; Mishu *et al.* 1992; Vandamme *et al.* 1992; Lerner *et al.* 1994; Lindblom *et al.* 1995; On *et al.* 1995; Kiss and Csorian 1996; Lauwers *et al.* 1996; Hsueh *et al.* 1997). In addition, many species are also implicated as causative agents of animal disease including abortion, enteritis, hepatitis and others (Garcia *et al.* 1983; Skirrow 1994; On 1996). In general, little is known of the epidemiology of these species and their clinical importance is probably underestimated since standard clinical laboratory isolation and identification techniques are not designed to detect or identify non-thermophilic campylobacters (Corry *et al.* 1995; On 1996). In addition, *Campylobacter* isolates are rarely identified to species in routine clinical laboratories; isolates are generally treated as *Campylobacter* spp. in surveillance data on food-borne infectious intestinal disease (Anon. 1997; Owen *et al.* 1997).

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Thermophilic *Campylobacter* spp. and (more recently) *Arcobacter butzleri* are common on poultry (Fricker and Park 1989; Flynn *et al.* 1994; Mead *et al.* 1995; Lammerding *et al.* 1996). Furthermore, we have recently detected two other species of *Arcobacter*, *A. cryaerophilus* and *A. skirrowii*, on poultry carcasses (Atabay *et al.* 1997). Both *A. butzleri* and *A. cryaerophilus* have been isolated from human gastrointestinal disease. *Helicobacter pullorum* has been isolated from intestinal contents of asymptomatic broiler chickens at slaughter and from the livers and intestinal contents of laying hens with vibrionic hepatitis (Stanley *et al.* 1994; Burnens *et al.* 1996). This species has also been associated with gastroenteritis in humans (Burnens *et al.* 1994).

We recently isolated a number of *Campylobacter*-like organisms from broiler chicken carcasses and faeces, but were at that time unable to identify them (Atabay and Corry 1997). This paper reports their identification as *H. pullorum* and provides further information on their prevalence on broiler chicken carcasses and in caecal contents.

MATERIALS AND METHODS

Twenty-six strains of bacteria that resembled campylobacters ('unclassified *Campylobacter*-like strains') but could not be identified to species level were isolated as described in detail by Atabay and Corry (1997). Briefly, carcass washings were made from (a) 10 chickens purchased from a national supermarket chain and (b) 15 chicken carcasses taken from the line in a poultry factory immediately after evisceration. The caecal contents from the latter 15 birds were also examined. Only fresh abattoir samples yielded this organism. The *Campylobacter*-like strains were isolated directly from carcass washings on blood agar by the method of Steele and McDermott (1984) but could not be detected after enrichment. They were isolated from the caeca both by direct plating and after enrichment. In addition, two chicken caecal strains isolated using a similar protocol on another occasion from a different flock and abattoir, were included in this study. One suspect colony from each colony type on each plate was picked, and checked by Gram stain, oxidase test and microscopic examination of wet mounts under phase contrast. Colonies giving reactions typical for campylobacteria were purified by subculturing on blood agar plates.

Phenotypic characterization of isolates

All isolates were characterized using the scheme of Bolton *et al.* (1992) and/or API Campy identification kits (Biomérieux, France).

Eighteen representative strains out of the 28 isolates (including the two isolated on another occasion) were selected on the basis of their API profiles (Table 1) and some phenotypic features obtained using the Bolton scheme. These strains were examined with a more comprehensive phenotypic identification scheme (On *et al.* 1996). However, anaerobic growth was assessed by use of a repeated evacuation procedure with a catalyst, as described previously for *A. nitrofigilis* (On and Holmes 1991a). In addition, isolates were tested for growth on blood agar under a microaerobic atmosphere without hydrogen at 37 °C. This atmosphere was achieved by completely evacuating an anaerobic jar without catalyst and re-filling it with a microaerobic gas mixture (10% CO_2, 5% O_2 and 85% N_2) three times. All tests were performed on freshly-prepared media, the quality of which had been tested using appropriate control strains, using the recommended procedures (On and Holmes 1991a,b, 1992, 1995; Bolton *et al.* 1992).

Computer-assisted identification of isolates

Phenotypic test results for each strain were compared with similar test data for 37 campylobacterial taxa (On *et al.* 1996) by use of a BASIC computer program, MATIDEN (Sneath 1979). Strains were considered identified if an identification score (Willcox probability) of 0·95 (i.e 95%) to a given taxon was obtained.

Preparation of protein samples, electrophoresis and staining

Whole-cell protein samples were prepared from 48–72 h bacterial cultures (Costas *et al.* 1993), subjected to electrophoresis (Atabay *et al.* 1998) and stained (Costas *et al.* 1993) as described previously. However, 16·6% polyacrylamide separation gels were used and proteins were electrophoresed at a constant voltage of 120 V throughout. Field isolates were identified by visually comparing their protein

Table 1 The code number (profiles) produced by *Helicobacter pullorum* strains with API Campy identification kits (total 28 strains)

Profile with API kits	*n**	Identification with API
0001004	8	'Not valid'
6003004	7	'Not valid'
2003004	5	Genus *Campylobacter*
2003000	2	*Campylobacter upsaliensis*
4001004	3	*Helicobacter fennelliae*
4003004	2	'Not valid'
4403004	1	'Doubtful profile'

**n*, Number of strains giving the same code number (profile).

banding patterns with those of reference strains of known taxa.

RESULTS

Examination of 28 strains of *Campylobacter*-like bacteria using the scheme of Bolton *et al*. (1992) and API Campy strips

All strains were Gram-negative, slightly curved, slender rod-shaped and motile under phase contrast microscopy. All were oxidase-positive, but urea, hippurate and indoxyl acetate were not hydrolysed and DNAase was not produced. The 26 strains isolated by Atabay and Corry (1997) all produced catalase, whereas the two isolates obtained on a different occasion were catalase-negative. All grew at 37 °C in a microaerobic atmosphere including hydrogen but none grew in a microaerobic atmosphere without hydrogen. They produced seven different code numbers with API Campy strips (Table 1). Most strains were not identified by the latter system; five were identified to genus level as *Campylobacter*, three as *H. fennelliae* and two as *Camp. upsaliensis*.

The results of the identification scheme of On *et al*. (1996)

Phenotypic characteristics of isolates. The results of these tests are shown in Table 2. Table 2 summarizes the tests in which all strains gave the same results and shows the tests in which all strains gave various results. Table 2 also includes the test results for 16 strains of *H. pullorum* reported by On *et al*. (1996) for comparisons with those given in this study.

Computer-assisted identification of isolates. Of the 18 strains tested using the phenotypic scheme of On *et al*. (1996), 14 were identified by the criteria defined previously (see Materials and Methods) as *H. pullorum*; two strains (DCC 7B2 and C12) achieved identification scores of 82·5% and 79·7%, respectively, to this species. Strain DCC 11B1 was not identified by this method, having attained an unacceptably low ID score (0·6 and 0·38, respectively) to the taxa *Camp. hyointestinalis* subsp. *lawsonii* and *H. pullorum*. The remaining strain (HDPCF5B) was misidentified to the *H. acinonyx–pylori* 'complex', despite its lack of urease and its ability to produce H_2S in TSI medium.

SDS-PAGE protein profiling of strains

The protein profiles of the 18 unusual H_2S-positive poultry isolates examined in detail were essentially indistinguishable from that of the reference strain of *H. pullorum* used (CCUG 33839), thus confirming their identity (Fig. 1). Moreover, *H. pullorum* strains were clearly distinguishable from other poultry and/or avian campylobacteria by this method (Fig. 1).

DISCUSSION

The results presented in this paper show that isolates previously called *Campylobacter*-like (Atabay and Corry 1997) are *H. pullorum*. These strains were found with other strains of *Campylobacter* and *Arcobacter* in nine of 15 chickens taken from a poultry abattoir (Atabay and Corry 1997; Atabay *et al*. 1998).

Accurate identification of helicobacters and related species (including *Campylobacter* and *Arcobacter* spp.) is known to be a difficult task (On 1996). Certainly *H. pullorum* poses a number of problems to workers striving to recognize it. The two traits that can be used as phenotypic markers for *Helicobacter* spp. (presence of sheathed flagella and resistance to polymyxin B) are absent in this species (Burnens and Nicolet 1993). Thus, *H. pullorum* strains may easily be mistaken for *Campylobacter* spp., particularly *C. coli* and *C. lari*, with which several key phenotypic traits are shared (cf. On *et al*. 1996). In addition, *H. pullorum* can be isolated from poultry and from humans with diarrhoea, both well-established sources of other campylobacteria. Furthermore, many workers are likely to be comparatively unfamiliar with this species, which was described relatively recently (Stanley *et al*. 1994). It is thus possible that *H. pullorum* isolates from host animals and human disease are being misidentified as *Campylobacter* spp., as illustrated in this study from the results obtained using API Campy identification kits (Table 1). The probability matrix used in this study (On *et al*. 1996) proved effective in identifying most isolates, although the need for careful inspection of the data was also evident. The identity of our strains was confirmed by protein profiling, a well-established identification method for campylobacteria (On 1996). Moreover, a polymerase chain reaction-based identification test is also available for *H. pullorum* (Stanley *et al*. 1994). These methods could be more widely used to confirm the identity of *H. pullorum* strains.

The misidentification of the strains using the API Campy and Preston identification schemes is not unexpected, because data for *H. pullorum* are not included in either of the aforementioned systems. Clearly these schemes should be updated to include important species of campylobacteria causing disease in humans and animals. Table 3 summarizes some phenotypic characteristics which are useful in distinguishing *H. pullorum* from other species of campylobacteria found on chickens and other birds. Differences were observed for some tests between the strains used in this study and those tested in the study of On *et al*. (1996). Examples include resistance to sodium arsenite and growth on potato starch and tyrosine (see Table 2 for details). These test results should therefore

Table 2 Phenotypic characteristics of the 18 field strains of *Helicobacter pullorum* compared with those of type and reference strains (cf. On *et al.* 1996) using the same test methods

Phenotypic characteristics	Percentage positive for strains from: This study*	The study of On *et al.* (1996)
100% positive or negative (this study)		
Gram-negative	100	99
Oxidase	100	99
Urease	0	01
Hippurate hydrolysis	0	01
DNAase	0	01
T.T.C. reduction	0	50
Selenite reduction	0	50
Trace H_2S in TSI	100	50
Acid and gas production in TSI	0	01
Indoxyl acetate hydrolysis	0	01
Green pigment	0	01
Blue-grey pigment	0	01
Alpha-haemolysis	100	99
Pitting on blood agar	0	01
Growth on special media		
T.T.C. (growth)	0	50
0·05% safranin	0	01
MacConkey	0	01
metronidazole/NA	0	01
campylobacter minimum medium	0	01
CCD		50
casein (growth)	0	01
basic fuchsin	0	25
crystal violet	0	01
janus green	0	06
pyronin/BA	0	01
pyronin/NA	0	01
methyl orange	0	99
sodium fluoride (NaF)	0	01
Growth at		
room temperature (mO_2)	0	01
37 °C (mO_2)	100	99
37 °C (mO_2 without H_2)	0	NA
37 °C (O_2)	0	01
25 °C (mO_2)	0	01
25 °C (O_2)	0	01
37 °C (AnO_2)/TMAO	100	01
Growth on media containing		
1·0% glycine	0	01
2·0% NaCl	0	01
3·5% NaCl	0	01
4·0% NaCl	0	01
Less than 100% positive or negative (this study)		
Catalase	89	88
Nitrate reduction	83	99
Alkaline phosphatase	11	01
Growth on media containing		
1·0% bile	72	88
1·5% bile	61	81
2·0% bile	56	81
Growth at		
42 °C (mO_2)	78	99
37 °C (AnO_2)	94	99
Growth on special media		
sodium arsenite ($NaAsO_2$)	67	01
0·02% safranin	6	01
nalidixic acid	28	06
cephalothin	89	99
metronidazole/BA	78	01
carbenicillin/BA	50	88
carbenicillin/NA	56	19
cefoperazone/BA	89	99
cefoperazone/NA	94	50
5-fluorouracil	50	69
potato starch (growth)	94	01
lecithin (growth)	39	06
tyrosine (growth)	61	01
sodium deoxycholate	17	01

* In total 18 strains were studied.
† Values of 0 and 100 were adjusted to 01 and 99 respectively to enable numeric comparison (see On *et al.* 1996 for details).
NA, Not available; mO_2 microaerobically plus H_2; O_2 aerobically; AnO_2 anaerobically; T.T.C., 2,3,5-triphenyltetrazolium chloride; BA, 5% horse blood agar; NA, nutrient agar; TSI, triple sugar iron agar; TMAO, trimethylamine-*N*-oxide; CCD, *Campylobacter* charcoal-deoxycholate medium (i.e. unsupplemented MCCDA).

in future be interpreted with caution. The scheme of On *et al.* (1996) will be amended by use of these data.

Details of the prevalence and methods of isolation of *H. pullorum* in 15 chicken carcasses and their caecal contents are given by Atabay and Corry (1997), where they were called '*Campylobacter*-like' strains. Nine of the 15 birds (originating from four different flocks) were positive for *H. pullorum* in the caecal contents and/or on the carcass. This indicates that *H. pullorum* is as common as *Camp. jejuni* subsp. *jejuni* in poultry. If this prevalence is confirmed by more extensive sampling, *H. pullorum* might be more important than previously realized in both human and animal diseases. *Helicobacter pullorum* has been isolated from poultry and from

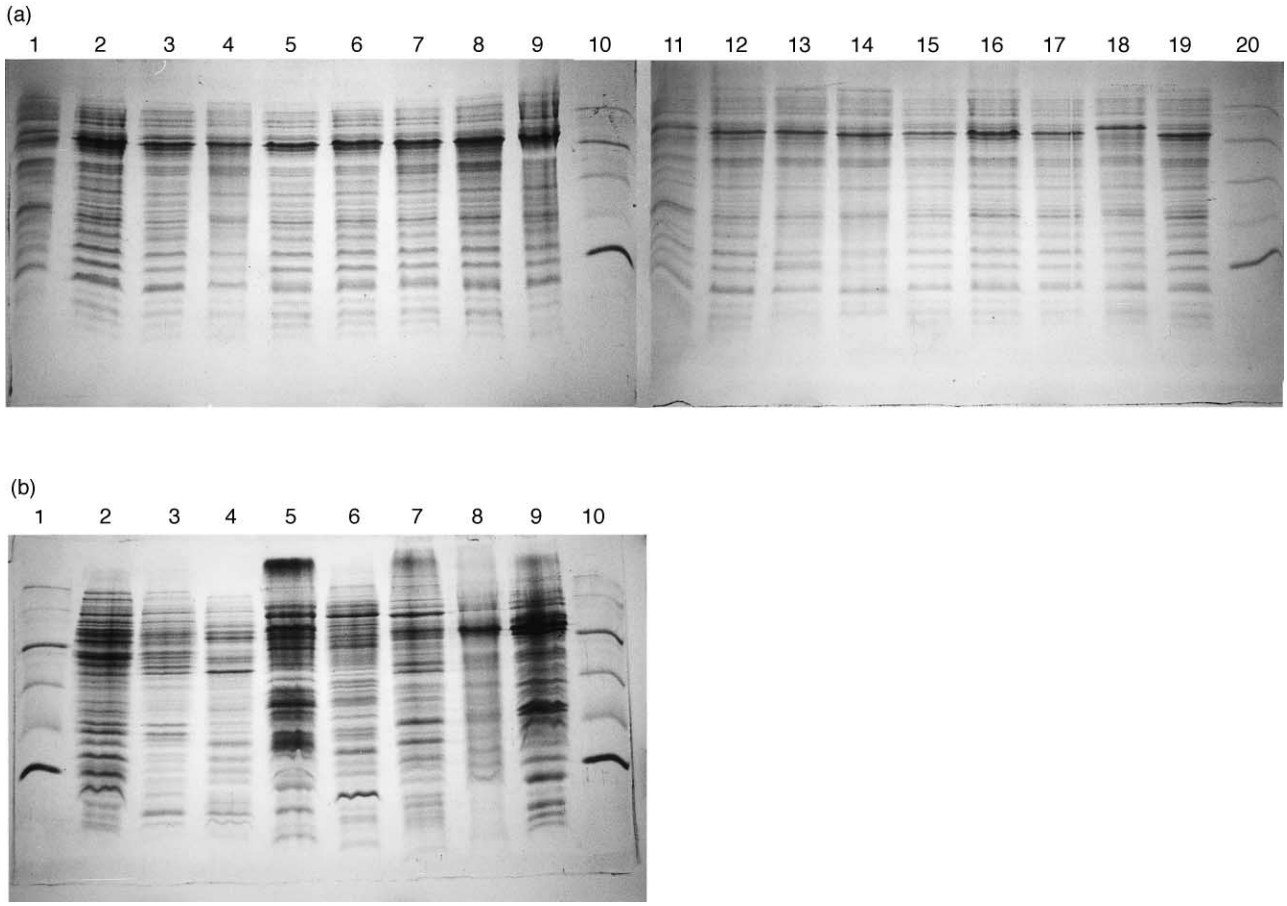

Fig. 1 Identification of poultry *Helicobacter pullorum* strains by comparative analysis of whole cell protein profiles. (a) Lanes 1–9 (DCC 11B, HDPCF5B, HCF 14, HCF 7, DCC 4B2, HCF 4, DCC 15B1, DCC 14B2 and CCUG 33839) and 11–19 (C12, DCC 8B2, HDPCF7B, HDPCF13B, HDPCF4B, DCC 5B2, DCC 7B1, C14 and DCC 13B1) are *H. pullorum* strains. (b) Lanes 2–3 (CF6 and CCUG 33839) are *H. pullorum* and 4–9 (CCUG 29255^{T}, *H. pamatensis*; CCUG 11284^{T}, *Campylobacter jejuni* subsp. *jejuni*; CCUG 23947, *Camp. lari*; CCUG 30485^{T}, *Arcobacter butzleri*; CCUG 17802, *A. cryaerophilus* and CCUG 10374^{T}, *A. skirrowii*) are type strains of other campylobacteria found in poultry and/or other avian species. Lanes 10 and 20 of (a) and 1 and 10 of (b) contain a molecular size marker (Bio-Rad, 161-0304) that comprised (all weights in kD): phosphorylase B, 107 000; bovine serum albumin, 76 000; ovalbumin, 52 000; carbonic anhydrase, 36 800; soybean trypsin inhibitor, 27 200 and lysozyme, 19 000. All CCUG strains are type(^{T}) or reference strains from the culture collection of the University of Göteborg, Sweden.

human patients with gastroenteritis. Very little information has been published concerning the prevalence of *H. pullorum*. In contrast with our study, detecting 60% of carcasses positive for *H. pullorum*, Burnens *et al.* (1996) reported that only 4% (six of 150) of intestinal contents of healthy broiler chickens were positive for this bacterium.

Helicobacter pullorum was first isolated using a selective medium containing 5% blood with cefoperazone (32 mg l^{-1}), vancomycin (10 mg l^{-1}) and amphotericin B (3 mg l^{-1}) (Stanley *et al.* 1994; Burnens *et al.* 1996). Although these bacteria do not require complex media, they do not grow on campylobacter selective media containing polymyxin B, as discussed above, and so would not grow on Blaser-Wang, Campy-BAP, Exeter, Skirrow and Preston selective media (Corry *et al.* 1995). In this study, *H. pullorum* strains were isolated only on non-selective blood agar using the membrane filtration method of Steele and McDermott (1984). Furthermore, our data show that *H. pullorum* does not grow on cefoperazone amphotericin teicoplanin or on modified cefoperazone charcoal deoxycholate agar media, nor in a microaerobic atmosphere lacking in hydrogen. The common use of these media, and of commercial kits used to generate conditions of microaerobiosis without hydrogen, will therefore not enable accurate estimates of the prevalence of *H. pullorum* in foodstuffs or human or animal disease to be made. We have also observed that the type strain of *H. pullorum* (NCTC 12824^{T}) grew poorly in broth culture media such as brucella broth and brain heart infusion broth (unpublished

Table 3 Probabilistic identification matrix for ampylobacteria (*Campylobacter*, *Arcobacter* and *Helicobacter* species) commonly found in poultry

Phenotypic characteristics	Per cent positive							
	H. pullorum	*Camp. jejuni*[1]	*Camp. coli*	*Camp. lari*[2]	*A. butzleri*	*A. cryaerophilus*[3]	*A. skirrowii*	*H. pamatensis*
Catalase	88	99	99	99	80*	97	99	99
Hippurate hydrolysis	01	99†	01	01	01	01	01	01
H_2S in TSI	76	01	50	01	01	01	01	01
Indoxyl acetate hydrolysis	01	99	99	07	99	99	91	01
Alpha-haemolysis	99	95	24	71	40	03	99	99
Selenite reduction	24	70	99	50	22	01	09	99
Alkaline phosphatase	06	01	01	07	01	01	01	80
Aerobic growth at 25 °C	01	01	01	01	99	99	99	01
mO_2 growth with added H_2 at 37 °C	99	99	99	99	99	76	99	99
Growth on media containing								
1% bile	79	99	99	99	99	76	99	01
1% glycine	01	90	94	99	01	01	01	60
2% NaCl	01	01	01	86	98	88	99	01
CMM	01	10	99	01	99	01	01	01
MacConkey	01	05	35	01	96	15	01	01
tyrosine	32	01	01	01	83	06	01	01

* Weak catalase reaction (oxygen was produced either in very small quantities or more than 10 s after H_2O_2 was added).
† Hippurate-negative strains were described (Totten *et al.* 1987).
Results for certain taxa include data: [1] *Campylobacter jejuni* subsp. *jejuni*; [2] nalidixic acid-sensitive *Campylobacter* and urease-positive thermophilic *Campylobacter* variants; [3] subgroups I and II.
mO_2, Microaerobic conditions; CMM, *Campylobacter* minimal medium; TSI, triple sugar iron medium.
Data were obtained from On *et al.* (1996); however, a combination of results from the study reported by On *et al.* (1996) and Atabay *et al.* (1998) was used for *Arcobacter* spp. For *Helicobacter pullorum* a combination of results from this study and those reported by On *et al.* (1996) were used. Data for *H. pamatensis* were obtained using the same test methods (S.L.W. On, published data). Values of 0 and 100 were adjusted to 01 and 99 respectively to enable numeric comparison (see On *et al.* 1996 for details).

observations). We also recommend an incubation temperature of 37 °C, since 22% of the strains did not grow at 42 °C when tested in this study (Table 2).

In conclusion, the true prevalence of *H. pullorum* in human and animal disease and in foodstuffs could hitherto have been masked for a number of reasons, including (a) its susceptibility to polymyxin B which is commonly used in many campylobacter isolation media and might impede the cultivation of these bacteria (Burnens *et al.* 1994; Corry *et al.* 1995), (b) its requirement for a hydrogen-containing atmosphere and (c) difficulties in its identification (Burnens *et al.* 1994; Stanley *et al.* 1994; also discussed here). Our data suggest this species to be relatively common in poultry and we recommend that suitable isolation and identification procedures be used to determine the true prevalence and importance of this organism.

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