Original article

Characteristics and antimicrobial resistance of *Campylobacter* isolated from pig and cattle carcasses in Poland

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Abstract

A total of 70 *Campylobacter* isolates recovered from 114 cattle and 177 pig carcasses at the slaughterhouse level were characterized by the presence of 7 putative virulence genes and antimicrobial susceptibility using the microbroth dilution method and minimal inhibitory concentration (MIC). The prevalence of *Campylobacter* was 14.9% and 29.9% in cattle and pig samples, respectively. The majority of cattle carcasses were contaminated with *C. jejuni* (64.7%), whereas pig carcasses were mainly positive for *C. coli* (77.4%). Most of the strain, irrespective of origin, possessed at least one pathogenic gene marker tested, mainly *flaA* and *cadF* genes responsible for motility and adherence to host epithelial cells, respectively. Several isolates also possessed the *cdtA* and *cdtB* genes responsible for the production of cytolethal distending toxin. Antibiotic profiling showed that campylobacters were most frequently resistant to quinolones (nalidixic acid and ciprofloxacin, total 57.1% of isolates) followed by streptomycin (52.9%, only *C. coli* strains) and tetracycline (51.4%). Resistance to erythromycin was demonstrated only in 4 *C. coli* strains of pig origin. None of the isolates, irrespective of origin, was resistant to gentamycin. Multi-resistance patterns, defined as resistance to antimicrobials of at least two different classes, were observed among 65.4% of the isolates, mainly *C. coli* recovered from pig carcasses.

Key words: *Campylobacter*, pigs, cattle, carcasses, virulence genes, antimicrobial resistance

Introduction

Campylobacter has been recognized as one of the most common causes of food-borne gastroenteritis in humans (Anon 2012). According to a recent European Food Safety Authority (EFSA) report, campylobacteriosis in the European Union is still the most common foodborne bacterial disease with 212,064 confirmed cases and a notification rate of 48.56 per 100,000 population in 2010 (Anon 2012). In Poland at the same time only 367 infections were notified with an incidence rate of 0.96 (Anon 2012). There is no information regarding how many of these infections were attributed to *Campylobacter* of cattle and pig origin. *Campylobacter* acts as a commensal in many food-producing animals such as cattle, pigs, and poul-

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try. Poultry are generally considered as the most important reservoirs for campylobacters, mainly *C. jejuni*, and as a primary source of human cases of campylobacteriosis (Mead et al. 1999, Keener et al. 2004, Anon 2012). On the other hand, cattle, and to a lesser extent pigs, are carriers of *Campylobacter* bacteria; however, there is little information concerning the contamination of their carcasses at the slaughterhouse level (Bae et al. 2007, Englen et al. 2007). As described by many authors, pigs carry a higher proportion of *C. coli* than *C. jejuni*; however, the carcasses are not as frequently contaminated with these bacteria as compared to poultry (Qin et al. 2011, Szygalski Biasi et al. 2011, Quintana-Hayashi and Thakur 2012). Recently, we have shown that bovine hides and carcasses tested in Poland at the slaughterhouse level were positive for *Campylobacter* at 25.6% and 2.7%, respectively (Wieczorek et al. 2013).

Several putative virulence markers have been described in *Campylobacter* isolates; however, the pathogenesis of infection is still not yet well defined (Müller et al. 2006, Fernandes et al. 2010). Pathogenic factors such as flagella-mediated motility (determined by the *flaA* gene), adherence to intestinal epithelial cells (*cadF* gene product), invasion and survival in the host cells (*iam* and *virB11* markers) as well as the ability to produce toxins (*cdt* genes) are important in the development of campylobacteriosis (Bang et al. 2003, Datta et al. 2003, Fernandes et al. 2010).

An increase in *Campylobacter* resistance, especially to quinolones (ciprofloxacin) and macrolides (erythromycin) as well as to other antimicrobials, has been recently observed (Endtz et al. 1991, Smole Možina et al. 2011). Food-producing animals, including cattle and pigs, may be a source of such resistant isolates that are transmitted to humans by food of animal origin (Aarestrup and Engberg 2001, Ghafir et al. 2007, Sheppard et al. 2009, Egger et al. 2012, Quintana-Hayashi and Thakur 2012). This applies particularly to strains resistant to quinolones and erythromycin, widely used for therapy in severe human infections or in immuno-compromised patients (Aarestrup and Engberg 2001).

The aim of the present study was to investigate the prevalence of *C. coli* and *C. jejuni* in cattle and pig carcasses at the slaughter level in Poland. The isolates were characterized by the identification of 7 different putative and toxin genes. Furthermore, the resistance of *Campylobacter* strains to several antimicrobials was also investigated.

Materials and Methods

Isolation and identification of *Campylobacter*

A total of 114 cattle and 177 pigs slaughtered during 2009-2011 in slaughterhouses located all over Po-

land were used in the study. The samples were essentially collected as described previously (Wieczorek and Osek 2010). Briefly, the carcasses were surface swabbed at the brisket area using sterile sponges. To each swab, 200 ml of Maximum Recovery Dilution (MRD, Oxoid, UK) was added and stomached for 3 min. After centrifugation at 1000 x *g* for 15 min, the pellets were re-suspended in 100 ml of selective enrichment Bolton broth plus 5% leaked horse blood and modified Bolton broth selective supplement (Oxoid) containing the following antimicrobials: vancomycin, cefoperazone, trimethoprim, and amphotericin B to prevent non-target microbials. The enrichment cultures were grown for 48 h at 41.5°C under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) and then plated onto Karmali agar (Oxoid) and *Campylobacter* blood free agar (Oxoid) with CCDA selective supplement (Oxoid) followed re-incubation under the previously described conditions for 48 h. Plates were examined for morphologically typical *Campylobacter* colonies which were confirmed by microscopic morphology, motility, microaerobic growth at 25°C and the presence of oxidase.

One bacterial isolate from each positive sample was tested using PCR. A bacterial colony was suspended in 1 ml of sterile water and centrifuged at 13,000 x *g* for 1 min. DNA was extracted using the Genomic-Mini kit (A&A Biotechnology, Poland) according to the manufacturer's instructions. *Campylobacter* species were identified using multiplex PCR (m-PCR) with three sets of primers specific for the simultaneous detection of the *C. jejuni* (the *mapA* gene target), *C. coli* (*ceuE* gene), and *Campylobacter*-specific *16S rRNA* gene as described previously (Wieczorek and Osek 2005). Furthermore, in the case of doubtful results, a second m-PCR was applied to identify the species-specific *hipO* and *23S rRNA* (*C. jejuni*), *glyA* (*C. coli, C. lari*, and *C. upsaliensis*), and *sapB2* (*C. fetus* subsp. *fetus*), respectively (Wang et al. 2002).

Detection of virulence genes

Campylobacter isolates were tested for the presence of the most often described virulence genes: *flaA, cadF, cdtA, cdtB, cdtC, iam*, and *virB11*. The PCR conditions for all genes were the same as previously described (Wieczorek 2010).

Antimicrobial susceptibility

A microbroth dilution method was used to establish the minimum inhibitory concentrations (MIC) of *Campylobacter* isolates to 7 antimicrobial agents using

Table 1. Antimicrobials, dilution ranges and cut-off values used for MIC determination of *Campylobacter*.

Antimicrobial class	Antimicrobials	Dilution range (mg/L)	Cut off values (mg/L)	
			C. jejuni	*C. coli*
Aminoglycosides	Gentamicin (GEN)	0.12 – 16	1	2
	Streptomycin (STR)	1 – 16	2	4
Macrolides	Erythromycin (ERY)	0.5 – 32	4	16
Quinolones	Nalidixic acid (NAL)	2 – 64	16	32
	Ciprofloxacin (CIP)	0.06 – 4	1	1
Tetracyclines	Tetracycline (TET)	0.25 – 16	2	2
Amphenicols	Chloramphenicol (CHL)	2 – 32	16	16

Table 2. Prevalence of *Campylobacter* isolated from pig and cattle carcasses.

Sample origin	No. (%) of samples			
		Positive for:		
	Tested	*Campylobacter*	*C. jejuni*	*C. coli*
Pigs	177	53 (29.9)	12 (22.6)	41 (77.4)
Cattle	114	17 (14.9)	11 (64.7)	6 (35.3)
Total	291	70 (24.0)	23 (32.9)	47 (67.1)

the Sensititre® custom susceptibility plates, EUCAMP (Trek Diagnostics, UK). Antimicrobials, dilution ranges, and cut-off values used for MIC determination are described in Table 1. The strains were sub-cultured twice on Columbia agar (Oxoid) at 41.5ºC for 48 h under microaerobic conditions. The minimum inhibitory concentration of the antimicrobial agents was determined using Mueller-Hinton Broth (Oxoid) supplemented with 2-2.5% horse blood (Trek). The plates were incubated at 37ºC for 48 h under microaerophilic conditions and read using the Vision® system (Trek). The antimicrobials and cut off values used for the interpretation of the MIC results were in accordance with EUCAST (www.eucast.org) and the European Union Reference Laboratory for Antimicrobial Resistance.

Reference strains

C. jejuni ATCC 33560, *C. coli* ATCC 43478, and *C. lari* ACTC 35221 were used in the study as reference strains. Furthermore, DNA isolated from *C. upsaliensis*, and *C. fetus* subsp. *fetus* obtained from the European Union Reference Laboratory for *Campylobacter*, Uppsala, Sweden were included as controls.

Statistical analysis

Statistical differences in the prevalence of virulence genes, *C. jejuni* and *C. coli* isolates recovered from pig and cattle carcasses, and in antimicrobial resistance were determined using a 2 x 2 contingency table and Fisher's exact test (Statistica, Poland). P values were two-tailed and groups were considered significantly different if P was < 0.05.

Results

Isolation and characterization of *Campylobacter*

During the study period, a total of 291 bovine and pig carcasses were examined for the presence of *Campylobacter*. Altogether, 70 (24.0%) samples were positive for this pathogen, including 53 pig (29.9%) and 17 cattle (14.9%) carcasses tested, respectively (Table 2). PCR analysis revealed that *Campylobacter* were identified either as *C. jejuni* (23 strains, 32.9%) or as *C. coli* (47 isolates, 67.1%). The majority of cattle carcasses were contaminated with *C. jejuni* (11 out of 17 samples, 64.7%; P>0.05) whereas pig carcasses were mainly positive for *C. coli* (41 out of 53 samples, 77.4%; P<0.001) (Table 2).

Table 3. Prevalence of virulence genes among Campylobacter isolated from pig and cattle carcasses.

Virulence gene	No. (%) of positive isolates				Total (n = 70)
	C. jejuni (n = 23)		C. coli (n = 47)		
	Pig (n = 12)	Cattle (n = 11)	Pig (n = 41)	Cattle (n = 6)	
flaA	11 (91.7)	11 (100)	40 (97.6)	6 (100)	68 (97.1)
cadF	10 (83.3)	11 100)	41 (100)	6 (100)	68 (97.1)
cdtA	11 (91.7)	11 (100)	36 (87.8)	5 (83.3)	63 (90.0)
cdtB	12 (100)	11 (100)	36 (87.8)	5 (83.3)	64 (91.4)
cdtC	12 (100)	11 (100)	11 (26.8)	2 (33.3)	36 (51.4)
iam	6 (50.0)	6 (54.6)	41 (100)	6 (100)	59 (84.3)
virB11	1 (8.3)	1 (9.1)	3 (7.3)	0	5 (7.1)

Table 4. Antimicrobial resistance of *Campylobacter* isolated from pig and cattle carcasses.

Antimicrobials	No. (%) of resistant isolates				Total (n = 70)
	C. jejuni (n = 23)		C. coli (n = 47)		
	Pig (n = 12)	Cattle (n = 11)	Pig (n = 41)	Cattle (n = 6)	
Gentamicin (GEN)	0	0	0	0	0
Streptomycin (STR)	0	0	33 (80.5)	4 (66.7)	37 (52.9)
Erythromycin (ERY)	0	0	4 (9.8)	0	4 (5.7)
Ciprofloxacin (CIP)	5 (41.7)	5 (45.5)	25 (61.0)	5 (83.3)	40 (57.1)
Nalidixic acid (NAL)	5 (41.7)	5 (45.5)	25 (61.0)	5 (83.3)	40 (57.1)
Tetracycline (TET)	3 (25.0)	2 (18.2)	28 (68.3)	3 (50.0)	36 (51.4)
Chloramphenicol (CHL)	0	0	0	0	0

Identification of virulence genes

Most of the strains, irrespective of the species and origin, possessed at least one pathogenic gene marker tested (Table 3). The vast majority of the isolates were positive for the *flaA* and *cadF* markers; most of them had *cdtA* and *cdtB* toxin genes. On the other hand, only 5 (7.1%) *Campylobacter* isolates were positive for the *virB* virulence marker. It was observed that all *C. coli* strains, both of pig and cattle origin, possessed the *iam* gene which was identified in ca. 50% of the *C. jejuni* isolates (P<0.001; Table 3). Furthermore, statistical differences in the prevalence of the *cdtC* gene subunit were observed among both bacterial species (P<0.001).

Antimicrobial resistance

The results of *Campylobacter* resistance to antimicrobials tested in this study are shown in Table 4. All strains were sensitive for gentamicin and chloramphenicol. Remarkable differences between *C. coli* and *C. jejuni*, especially in resistance to streptomycin (P<0.001) and tetracycline (P<0.05), were observed. It was found that all *C. jejuni* isolates were sensitive for streptomycin, whereas as many as 80.5% and 66.7% *C. coli* strains of pig and cattle origin, respectively, were resistant to this antimicrobial. In the case of tetracycline, the differences were less pronounced, but again more *C. coli* than *C. jejuni* were resistant (Table 4). Similar observations were made for quinolones (nalidixic acid) and fluoroquinolones (ciprofloxacin). Only 4 isolates (*C. coli* of pig origin) were resistant to erythromycin.

Multi-resistance, defined as resistance to antimicrobials belonging to at least two different classes of antibiotics, was found among 43 out of 70 (61.4%) *Campylobacter* tested (Table 5). The highest level of resistance was observed in relation to quinolones and fluoroquinolones (nalidixic acid and ciprofloxacin) together with aminoglycosides (streptomycin) and tetracyclines (13 isolates in total, all of them *C. coli*). Several strains were also resistant to streptomycin

Table 5. Multiantimicrobial resistance of *Campylobacter* tested in the study.

| Resistance pattern | No. (%) of resistant isolates | | | | Total (n = 70) |
| | *C. jejuni* (n = 23) | | *C. coli* (n = 47) | | |
	Pig (n = 12)	Cattle (n = 11)	Pig (n = 41)	Cattle (n = 6)	
STR+TET	0	0	10	0	10
CIP+NAL+ERY	0	0	1	0	1
CIP+NAL+TET	1	1	3	1	6
CIP+NAL+STR	0	1	6	2	9
CIP+NAL+STR+TET	0	0	12	1	13
CIP+NAL+STR+TET+ERY	0	0	3	1	4

STR, streptomycin; TET, tetracycline; CIP, ciprofloxacin; NAL, nalidixic acid; ERY, erythromycin.

and tetracycline (10 *C. coli* of pig origin) and to quinolones and streptomycin (9 isolates, most of them *C. coli*) (Table 4). It was found that 4 *C. coli* strains displayed the multiresistance pattern to 4 antimicrobial classes consisting of 5 antibiotics (Table 4).

Discussion

A total of 70 *Campylobacter* isolates were recovered from 291 bovine and porcine carcass samples at the slaughterhouse level. Most of the strains were of pig origin contaminated at the 29.9% level whereas only 14.9% of cattle samples were positive for *Campylobacter*. There is little information concerning the prevalence of *Campylobacter* on carcasses of meat producing animals. Generally, the available data show that occurrence of these bacteria in pig samples collected at slaughterhouses was low, especially when compared with samples of poultry origin (Mead et al. 1999, Keener et al. 2004, Ghafir et al. 2007, Anon. 2012). The prevalence of *Campylobacter* in pig carcasses found in the present study was higher than the frequency of 0-5% identified in studies in Japan, Austria, and the United Kingdom (Ono and Yamamoto 1999, Mayrhofer et al. 2004, Little et al. 2008). On the other hand, Ghafir et al. (2008) reported 17% of pig carcasses positive for *Campylobacter* tested from 1997 to 1999 in Belgium. Recently, Szygalski Biasi et al. (2011) identified a high level of swine *Campylobacter*-positive carcasses (18.9%) but only 37 animals were investigated. A broader investigation performed in Alberta, Canada with 1,070 pork carcasses sampled at the slaughterhouse level with a swab method revealed that 8.8% of animals were positive for these bacteria (Bohaychuk et al. 2011). Furthermore, baseline surveys of pre-chill pork carcasses in Sweden (Lindblad et al., 2007) and Taiwan (Yeh et al. 2003) found that 1.0% and 13.8% of samples, respectively, were con-

taminated with *Campylobacter* spp. All these results, including the present investigation, indicate a wide variation in reported positive rates for pig carcasses tested.

As shown in the present study, contamination of bovine carcasses (14.9%) was much higher than identified in other surveys. In the previously mentioned study of Bohaychuk et al. (2011) only 1.5% of similar samples were positive for *Campylobacter*. Low contamination rates (3.5% and 3.3%) of bovine surface samples at the slaughterhouse level was also found in Finland and Belgium, respectively (Ghafir et al. 2007, Hakkinen et al. 2007). However, there are also other studies where prevalence rates of *Campylobacter* at slaughter between of 7% and 100% have been reported (Garcia et al. 1985, Stanley et al. 1998, Beach et al. 2002, Bae et al. 2005, Englen et al. 2007, Quintana-Hayashi and Thakur 2012). Recently, we have shown in another study that bovine carcasses were contaminated at a relatively low level (2.7% of positive swab samples) (Wieczorek et al. 2013). However, it is difficult to compare all the results described by us and other authors due to different study protocols, including sampling stages and *Campylobacter* detection methods.

Overall, of the 70 *Campylobacter* isolates identified in the present study, most were *C. coli* (67.1%), mainly due to contamination of pig carcasses (77.4% positive samples). These results are in agreement with other studies where bovine and porcine carcasses were also predominantly positive for *C. jejuni* and *C. coli*, respectively (Bae et al. 2005, Englen et al. 2007, Ghafir et al. 2007, Hakkinen et al. 2007, Wieczorek et al. 2013).

In human campylobacteriosis *C. coli* is reported less frequently than *C. jejuni*. According to a recent EFSA report (Anon. 2012), the most frequently identified *Campylobacter* species in 2010 was *C. jejuni* (35.7%) which was responsible for the majority

of human infection cases (93.4%) and only 2.3% of confirmed infections were due to *C. coli*. However, as many as 51.8% of the 212,064 *Campylobacter* isolates were not characterized at the bacterial species level. Case-control studies suggest that etiological risk factors for human infection with these two species are different but *C. coli* may play a considerably more important role than previously thought (Gillespie et al. 2002, Tam et al. 2003, Leatherbarrow et al. 2004).

Campylobacter isolated in the present study were characterized for the presence of virulence and toxin genes. Generally, the prevalence of putative virulence markers was similar to other data, although it is difficult to compare the results due to different genes examined, PCR primers used, and number and origin of the samples. Almost all *Campylobacter* strains possessed the *flaA, cadF, cdtA, and cdtB* markers which is in agreement with the results of several other studies (Bang et al. 2003, Datta et al. 2003, Rozynek et al. 2005, Müller et al., 2006, Krukiewicz and Klimuszko 2010, Wieczorek 2010). The prevalence of these genes was quite similar in *C. coli* and *C. jejuni*, irrespective of the strain's origin. However, due to a relatively low number of isolates it is difficult to assess whether the pathogenic potential of both *Campylobacter* species was similar.

It was found that *Campylobacter* strains identified in the present study were most commonly resistant to quinolones (ciprofloxacin and nalidixic acid, both 57.1% of the isolates) although more *C. coli* than *C. jejuni* displayed this resistance pattern. Furthermore, over 50% of the isolates were resistant to streptomycin (only *C. coli*) and tetracycline (both *C. coli* and *C. jejuni*). Several studies of antimicrobial resistance of *Campylobacter* recovered from cattle and pigs showed different results. Englen et al. (2007) investigated 473 *C. jejuni* strains from dairy cattle and only 2.5% were resistant to CIP. On the other hand, none of the 59 *C. coli* was resistant to this antimicrobial. In the case of tetracycline as many as 47.4% and 66.1% of *C. jejuni* and *C. coli*, respectively, were resistant to these antibiotics. In a similar study performed by Bae et al. (2005) only 5.1% *C. jejuni* strains were resistant to quinolones. Furthermore, *C. coli* of the same origin were more frequently resistant than *C. jejuni*, i.e. 45.5% of the isolates displayed the quinolone resistance pattern. On the other hand, only 5.9% and 1.1% of *C. jejuni* recovered from cattle in Finland were resistant to quinolones and tetracycline, respectively (Hakkinen et al. 2007).

Several studies described the resistance of *Campylobacter* isolates recovered from pigs. Most of the strains were usually resistant to tetracyclines and erythromycin. This is of public health interest because macrolides (ERY) as well as quinolones (CIP) are often chosen for treating severe cases of campylobacteriosis in humans (Aarestrup and Engberg 2001). Thakur and Gebreyes (2005) found that 66.2% of *C. coli* isolates of pig origin were resistant to tetracycline and 53.6% to erythromycin. A similar study performed by Shin and Lee (2007) revealed that resistance rates to these antimicrobials were 56.1% and 46.5%, respectively. The results of a recent survey by Egger et al. (2012) on the prevalence and antibiotic resistance of *C. coli* recovered from fattening pigs showed that 33.6% of the isolates were resistant to quinolones whereas 10.6% displayed resistance to macrolides (erythromycin). A broad study performed with *C. coli* of pig origin in China also identified a high level of resistance to ciprofloxacin and tetracycline (both from 95.8% to 99.0%) and erythromycin (37.9 – 54.7%) (Qin et al. 2011). Previous studies performed in Poland revealed that among nine pig *Campylobacter* isolates three were resistant to erythromycin, four to ciprofloxacin, and six to tetracycline. However, it is difficult to compare these results with the present investigation due to the low number of strains tested by Krutkiewicz et al. (2009).

Multi-antibiotic resistance (MAR), defined as resistance to antimicrobials of 2 or more different classes, was often detected in the isolates tested in the present study (61.4%) and it was more common among *C. coli* than *C. jejuni* isolates. Furthermore, the *C. coli* strains of pig origin showed a higher resistance than those recovered from cattle carcasses. MAR strains of cattle origin were also reported by Bae et al. (2005), but with a much lower percentage of quinolone-resistant strains, especially *C. jejuni* isolates (5.1%). Similar results were obtained by Inglis et al. (2004), who identified less than 1% of *Campylobacter* ciprofloxacin-resistant strains of cattle origin. In general, antibiotic resistance, including MAR, in *Campylobacter* recovered from these food-producing animals is much less frequent than in those isolated from poultry and pigs (Englen et al. 2007, Rozynek et al. 2008, Wieczorek 2010, Szygalski Biasi et al. 2011). However, in the present study most of the multi-resistant isolates were of *C. coli* and they were predominantly found in pigs. Recently, MAR of *Campylobacter* isolates identified in cattle hides and carcasses in Poland was also analyzed but fewer such strains (only 30 out of 115; 26.1%) than in the present investigation were detected (Wieczorek et al. 2013). On the other hand, Qin et al. (2011) identified a high proportion (76.8%) of *C. coli* isolates originating from pigs in China displaying MAR patterns, predominantly resistant to quinolones, kanamycin,

and tetracycline (89.5% of the 190 strains) as well as to quinolones, macrolides, and tetracycline (71.4%). A similar pattern (CIP, NAL, STR, TET, ERY) was identified in only 4 (5.7%) of the *Campylobacter* (all *C. coli*) strains in the present study. Another study performed by Shin and Lee (2007) with *C. coli* of pig origin in Korea also revealed a high percentage (56.1%) of MAR isolates, especially resistant to CIP and TET (77.2%). Such differences between results obtained in Poland and those two countries may be due to different preferences in regard to the use of antimicrobials for disease treatment or to enhance the growth of animals, or the methods applied for *Campylobacter* isolation and antimicrobial resistance testing.

In conclusion, cattle and pigs may play a role as an underestimated reservoir of potentially pathogenic *Campylobacter* strains for humans. These animals could contribute to human campylobacteriosis cases and outbreaks through consumption of contaminated meat. The results obtained also provide evidence that antimicrobial resistance is common among *Campylobacter* strains isolated from cattle and pigs in Poland, thus indicating the need for continued monitoring and application of reduction strategies within these meat producing animals.

Acknowledgements

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