

Full Length Research Paper

Comparison of Norwegian poultry, waterborne and clinical isolates of *Campylobacter jejuni* by ribotyping

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Campylobacteriosis is the most frequently occurring food and waterborne gastroenteritis in Norway and 90% of cases are caused by *Campylobacter jejuni*. Campylobacteriosis is epidemiologically associated with use of non-disinfected water and consumption of contaminated poultry. In order to provide molecular epidemiological support for this association isolates from local poultry and water were compared with human isolates from domestic and import infections using ribotyping. Automatic riboprints were performed with the *PstI* restriction enzyme and RiboPrinter® and compared with a library of recognised patterns (DUP-IDs). Patterns were further compared with each other using GelCompar software. The isolates from human clinical cases showed high heterogeneity. DUP-IDs found among human isolates were also found in 2 isolates from poultry, DUP-PSTI-1146 and DUP-PSTI-2061. Two human isolates were like isolates from water, DUP-PSTI-2073 and DUP-PSTI-1122, but these were from patients infected abroad. This study provides limited support for the importance of poultry as a source of infection and illustrates the need for studies of much larger scope to encompass the huge diversity of strains and sources of *C. jejuni*.

Key words: *Campylobacter*, similarity, clustering, genotyping, riboprint.

INTRODUCTION

The incidence of human cases of *Campylobacter* infection has increased and recently exceeded that of *Salmonella* in many European countries (<http://www.efsa.europa.eu/en.html>) and in the United States (Altekruse et al., 1999) and is the most frequently reported cause of bacterial gastrointestinal illness. Interest has been focused on the identification of risk factors and effective control measures. The pathogenic thermophilic campylobacters, represented by *Campylobacter jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*, are zoonotic with many animals serving as reservoirs for human diseases including rabbits, rodents, wild birds, sheep, horses, cows, pigs, poultry and domestic pets (Rosef et al., 1983; Kapperud and Rosef, 1983; Brown et al., 2004; Johnsen et al., 2006a). Although much work has been done to identify the environmental and animal reservoirs

of *Campylobacter* knowledge of pathogenesis and epidemiology is still incomplete. Case-control studies have identified a range of different risk-factors for infection and consumption of chicken is the most frequently reported risk-factor (Stafford et al., 2008). In industrialized countries, cases of human campylobacteriosis are about 90% due to *C. jejuni* and 10% due to *C. coli* (Kapperud, 1994).

Campylobacter are apparently fragile organisms that in the environment are unable to grow in the presence of air or multiply outside warm-blooded hosts and are highly susceptible to a number of environmental conditions (Park, 2002). Murphy et al. (2006) summarize the mechanisms by which *Campylobacter* spp. adapt to stress conditions and thereby increase their ability to survive on food and in the environment.

In addition to epidemiological studies, reliable and powerful typing methods for comparing campylobacters are necessary in order to gain more insight into infection routes. Traditionally, phenotyping methods such as serotyping and biotyping have been used. The drawbacks of these methods are their restricted resolution, the lack of

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specific reagents for serotyping and a large proportion of untypable strains. Using genotyping to determine genetic relatedness is necessary to provide data for a better understanding of the epidemiological aspects. Broman et al. (2004) found a high degree of diversity among *C. jejuni* isolates from migrating birds with pulse-field gel electrophoresis (PFGE) using the restriction enzymes *SmaI* and *KpnI*. A high degree of diversity was found in outdoor environmental isolates (Johnsen et al., 2006a) and among isolates from cattle (Johnsen et al., 2006b) by using *BglII* and *MfeI* and amplified fragment length polymorphisms (AFLPs). Ge et al. (2006) used both PFGE with the restriction enzyme *SmaI* and ribotyping with *PstI* for genotyping and found genetic diversity among isolates from retail meats. They found similarity among human and environmental strains. Multilocus sequence typing (MLST) has been used by Lévesque et al. (2008) to investigate the genetic diversity among *C. jejuni*. Nielsen et al. (2000) compared Ribotyping to other typing methods and showed it to be intermediate in discrimination with good correspondence with other methods. It has the advantage that the suppliers provide a continually updated database of patterns (DUP-ID) for the restriction enzyme *PstI*, allowing the patterns identified to be equated to those found by other users of the RiboPrinter®.

In Norway and other developed countries contaminated water, poultry meat and contact with domestic animals have been identified as risk factors for campylobacteriosis (Kapperud et al., 1992; Bryan and Doyle, 1995, Blaser, 1997; Rautelin and Hänninen, 2000; Kapperud et al., 2003). In this study we use automated ribotyping (Ribotyping) to compare *C. jejuni* isolates from poultry, river water and humans infected in Norway and abroad in order to investigate whether the epidemiological data can be corroborated by molecular typing.

MATERIALS AND METHODS

Bacterial strains

Clinical *C. jejuni* isolates from patients with diarrhoea were from stool samples sent to Unilabs Telelab for routine analysis. Patients were from the county of Telemark or from adjoining regions of the neighbouring county of Vestfold. Samples were spread on Preston agar plates (Mast, Merseyside, UK) and incubated in a micro-aerophilic atmosphere for 48 h at 40°C. *C. jejuni* were identified by colony morphology, motility, oxidase test and hippurate degradation. Isolates were randomly selected among 108 *C. jejuni* isolated in the period March to September 2008 according to the following criteria. Samples lacking clinical information on symptoms or where the patient had been infected were excluded. Only one sample per patient was included. Equal (± 2) numbers of isolates from patients infected in Norway and abroad were selected. Among the latter group, equal numbers of infections from Europe, America, Africa and Asia were chosen. Twenty two isolates were from domestic infections while 20 were infected abroad. 34 *C. jejuni* were from 2 poultry flocks in Telemark isolated over a period of 2 months in 2006 and 17 isolates were collected throughout 2006 from Bø river in Telemark County. The isolates were kept frozen at -70°C in Microbank™ (Pro-Lab Diagnostics, Canada) and re-cultivated on

on CCDA agar (Oxoid Ltd, Basingstoke, Hampshire, United Kingdom) for further examination.

PCR-assay

Species identification by multiplex PCR

The colony multiplex-PCR test described by Wang et al. (2002) was used for species confirmation. This test generates PCR products of characteristic length from the *hipO* gene of *C. jejuni*, the *glyA* gene of *C. coli*, *C. lari* and *C. upsaliensis* and the 23s RNA gene of all these species, which serves as an internal control. A loopful of material from a single colony was suspended in 500 µl of water, heat treated at 100°C for 10 min and centrifuged. 5 µl of the supernatant was used as a template. PCR Reactions were run on the iCycler (BioRad, Hercules, CA) in a 50 l volume using QIAGEN Multiplex PCR mastermix (QIAGEN, Hilden, Germany). The PCR program was: 15 min sample denaturation at 95°C, followed by 30 cycles of {94°C, 30s; 56°C, 90s; 72°C, 60s}, then 72°C, 7 min after which the samples were cooled to 4°C until analysis. This is a reoptimised protocol, differing from that described by Wang et al. (2002) in having a longer sample denaturation step, lower denaturation temperature, a longer annealing step at a lower temperature and longer extension time. PCR products were separated by agarose gel electrophoresis in 0.5xTBE at 135V for 1 hour on a 1.5% agarose gel, stained with ethidium bromide (Maniatis et al., 1982) and imaged under UV illumination using Gene Genius gel documentation equipment (Syngene, Cambridge, UK). Previously characterised *C. coli*, *C. jejuni*, *C. lari* and *C. upsaliensis* strains were used as positive controls and reference standards.

Ribotyping

Ribotyping was performed using the DuPont (Wilmington, DE) Qualicon RiboPrinter®. Single colonies from a 24 h culture on blood agar plates were suspended in sample buffer and heated at 80°C for 15 min. After the addition of lytic enzymes, samples were transferred to the RiboPrinter®. Further analysis for the restriction of DNA, including use of the *PstI* enzyme (20.000 U/ML, New England Biolabs Inc., Ipswich, MA), was carried out automatically. The ribo-print profiles were aligned according to the position of a molecular size standard and compared with patterns stored in the DuPont Identification library of the RiboPrinter®. The identification (DUP-ID) of an isolate was called when the corresponding patterns matched one of the library patterns with a similarity of > 0.85. The *PstI* ribo-type patterns were automatically assigned a DuPont identification number (e.g. DUP- PST1-1182) by the RiboPrinter®, which was confirmed by visual inspection. The ribotype profiles were further analyzed with the GelComparII® software (Bio Numerics, Applied Maths Inc., Gent, Belgium) using the Pearson correlation and default settings for optimization (2.0%) and position tolerance (1.00%) for genetic similarity and dendrograms were generated by the unweighted pair group method with arithmetic averages (UPGMA). These isolate-to-isolate comparisons were used to define "ribogroups". The similarity threshold for an isolate joining a ribogroup is an adaptive value between 0.90 and 0.96, depending on the size of the ribogroup.

RESULTS

Multiplex PCR confirmed all isolates as *C. jejuni*. Isolates from human cases infected in (I) or outside Norway (U) showed high heterogeneity (Figure 1). 25 of the isolates (60%) were assigned 18 different DUP-IDs from the

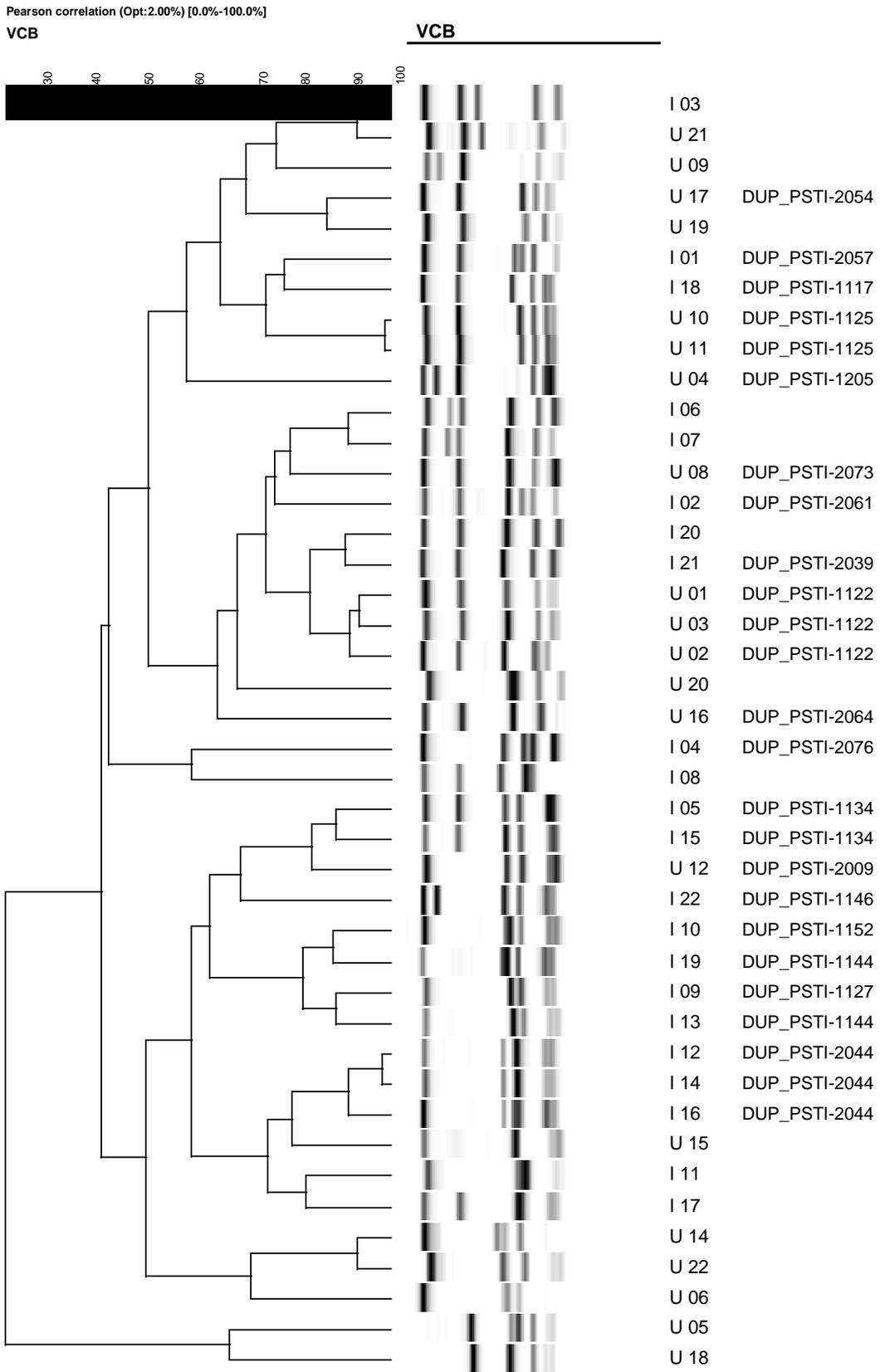


Figure 1. Dendrogram of 42 *Campylobacter jejuni* isolates from patients with diarrhoea infected in Norway (I) and abroad (U).

library. 3 isolates were DUP-PSTI-1122 (U) and DUP-PSTI-2044 (I) 2 isolates were DUP-PSTI-1125 (U), DUP-PSTI-1134 (I) and DUP-PSTI-1144(I) (Figure 1). Single occurrences of DUP-PSTI-1117, 1127, 1146, 1152, 2039, 2057, 2061 and 2076 and of DUP-PSTI-1205, 2009, 2054, 2064 and 2073 were found among the domestic and foreign isolates respectively. One patient had acquired campylobacteriosis caused by DUP-PSTI-1125 in Denmark and another in Thailand. Of the 17 isolates from water 4 could be assigned a DUP-ID (DUP-PSTI-1130, 1122, 2000 and 2073). Strains DUP-PSTI-2073 and DUP-PSTI-1122 were found among the both human water isolates as shown in Figure 2 (subcluster c). Of 34 isolates from poultry 10 (29%) were assigned a DUP-ID (DUP-PSTI-1122, 1125, 1205, 2009, 2054, 2064 and 2073), 2 of which, DUP-PSTI-1146 and DUP-PSTI-2061 were also found among the human strains as shown in Figure 2 (subclusters a and c). 14 of the closely related strains in cluster c were isolated from poultry. A similarity of human and water isolates was found in all subcluster (a - e), while similarity between human and poultry was found in subcluster a and c (Figure 2).

DISCUSSION

The majority of the developed countries have experienced an increase in human campylobacteriosis during the last 20 years and it is currently the most frequently reported bacterial enteric infection in many developed countries, including Norway with 2875 reported cases in 2008 (MSIS report 2008). The incidence of campylobacteriosis in Telemark County was 41.2 per 100.000 while the national incidence was 59.8. Most cases of campylobacteriosis are sporadic. In Norway, as in other developed countries, studies of outbreaks as well as case-control studies have identified contact with animals, outdoor barbecuing, consumption of contaminated water, poultry meat and contaminated ready-to-eat foods as significant risk factors for acquiring campylobacteriosis (Kapperud et al., 1992; Bryan and Doyle, 1995; Blaser, 1997; Rautelin and Hänninen, 2000; Kapperud et al., 2003). The isolates from the human sources in this study showed a high heterogeneity. It was not possible to trace the clustered isolates from Norway, DUP-PSTI-1144 (I) and DUP-PSTI-2044 (I) to outbreaks. The high diversity of strains giving campylobacteriosis makes it difficult to identify virulent strains.

Campylobacters are frequently isolated from water and water supplies and have been the cause of infection in reported outbreaks in many countries including Norway (Mentzing, 1981; Vogt et al., 1982; Rosef and Mork, 1985; Dahl and Melby, 1987; Melby et al., 1991; Koenraad et al., 1997). Waterborne outbreaks associated with contaminated drinking water are common in the Nordic countries (Hänninen et al., 2003). In an earlier study, Rosef et al. (2001) isolated *C. spp.* in 53.3% of water samples from the Bø River. High degree of contamination

has also been reported in Finland (Hörman et al., 2004) and New Zealand (Savill, 2001). Waterfowl are regular visitors to most surface water. Because of a higher body temperature, which is favourable for campylobacters, birds have different faecal flora than mammals and normally a lower content of faecal coliforms. 2 waterborne outbreaks of campylobacteriosis caused by geese (*Anser brachyrhynchus*) in Norway are described by Varslot et al. (1996). It is likely that contamination from birds plays a major role in contamination and transmission routes into water. Case-control studies have revealed non-disinfected drinking water and barbecuing as health hazards (Kapperud et al., 1992).

Domestic animals and wild birds may effectively spread *Campylobacter* by contaminating surface water sources. In many parts of the developed and developing world, un-disinfected surface water is used for human consumption. The isolates in this study represent surface river water. A prospective case – control study suggest that the waterborne route of infection may be the common underlying pathway linking infection in humans, poultry, other domestic animals and wild birds (Kapperud et al., 2003). We found similarity of isolates from water and humans infected abroad with ribotypes, DUP-PSTI-1122 and DUP-PSTI-2073. Isolates from both human and water were found in subclusters a – e as shown in the dendrogram (Figure 2). Automatic ribotyping to identify isolates from a waterborne outbreak has been used (Nielsen et al., 2000) and shown to be a good tool for epidemiological studies. A study of ribotypes of *C. jejuni*, *C. coli* and *C. lari* from water formed the strains in a main cluster. These isolates are normally distributed among birds (Rosef et al., 2009).

Broiler fowl are commonly colonized by *Campylobacter*, being symptomless carriers of the organism (Newell and Fearnley, 2003). The reported prevalence varies from 5% (Hofshagen and Kruse, 2005) in Norway to 90% in Germany (Atanossava and Ring, 1999). Infected broilers have high levels of *Campylobacter* in the intestines (Newell and Fearnley, 2003) and large diversity has been found between and within broiler flocks (Petersen et al., 2001). One infected flock in this study showed strain stability, Figure 2, subcluster c. Poultry carcasses contaminated through the slaughtering process represent a risk of human infection. We found 2 poultry strains, DUP-PSTI-1146 and DUP-PSTI-2061 similar to human isolates acquired locally. The similar isolates were found in subcluster a and c (Figure 2).

The finding that 2 locally acquired human infections were caused by strains with ribotype patterns identical to poultry isolates provides tentative support for the epidemiological evidence linking contaminated poultry to campylobacteriosis. On the other hand, 3 of the 4 human infections which were similar to water-borne isolates were acquired abroad and most of the human isolates were not similar to any of the poultry or water isolates. This may be a consequence of the limited scale of this study. The

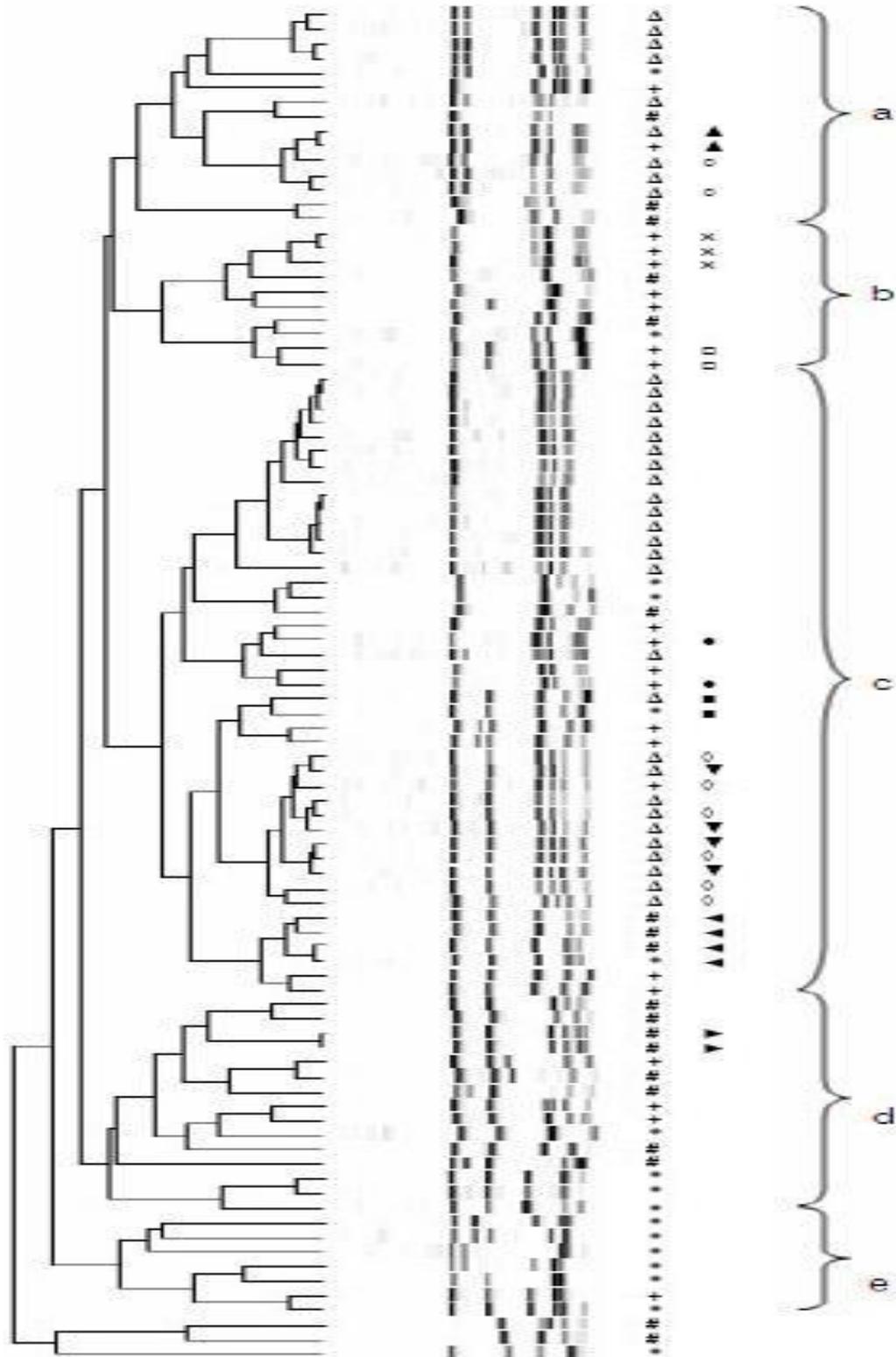


Figure 2. GelCompar dendrogram of riboprint patterns for poultry (), water (*) and human *C. jejuni* isolates from domestic (+) and foreign infections (#). Recognised riboprint patterns (DUP-ID) occurring more than once are DUP-PSTI-1146 (), DUP-PSTI-2050 (), DUP-PSTI-2044 (x), DUP-PSTI-1134 (), DUP-PSTI-1144 (), DUP-PSTI-2073 (), DUP-PSTI-2061 (), DUP-PSTI-1131 (), DUP-PSTI-1122 () and DUP-PSTI-1125 ().

poultry and water isolates encompassed 2 poultry farms and 1 river while the catchment area for clinical samples was the entire county of Telemark and the proximal part of the neighbouring county of Vestfold. The great diversity of isolates and potential sources means that a study on a much larger scale would be required to definitively corroborate the epidemiological findings.

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