

Characterization of a haemolytic phospholipase A₂ activity in clinical isolates of *Campylobacter concisus*

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A membrane-bound, haemolytic phospholipase A₂ (PLA₂) activity was detected in clinical strains of *Campylobacter concisus* isolated from children with gastroenteritis. The clinical strains were assigned into two molecular groups (genomospecies) based on PCR amplification of their 23S rDNA. This calcium-dependent, heat-stable, haemolytic PLA₂ activity was detected in strains from both genomospecies. A crude haemolysin extract (CHE) was initially prepared from cellular outer-membrane proteins of these isolates and was further fractionated by ultrafiltration. The haemolytic activity of the extracted fraction (R30) was retained by ultrafiltration using a 30 kDa molecular mass cut-off filter, and was designated haemolysin extract (HE). Both CHE and HE had PLA₂ activity and caused stable vacuolating and cytolytic effects on Chinese hamster ovary cells in tissue culture. Primers for the conserved region of *pldA* gene (phospholipase A gene) from *Campylobacter coli* amplified a gene region of 460 bp in all tested isolates, confirming the presence of a homologous PLA gene sequence in *C. concisus*. The detection of haemolytic PLA₂ activity in *C. concisus* indicates the presence of a potential virulence factor in this species and supports the hypothesis that *C. concisus* is a possible opportunistic pathogen.

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INTRODUCTION

Campylobacter species are one of the most common causes of human gastroenteritis worldwide. *Campylobacter jejuni* and *Campylobacter coli* are the species most commonly involved (Leach, 1997). However, recent studies have suggested that *Campylobacter* species other than *C. jejuni* and *C. coli*, such as *Campylobacter concisus*, *Campylobacter curvus*, *Campylobacter gracilis*, *Campylobacter upsaliensis* and *C. jejuni* subsp. *doylei* (Musmanno *et al.*, 1998; Maher *et al.*, 2003), may account for a proportion of cases of acute gastroenteritis in which no aetiological agent was identified.

In the early 1990s, there was an increase in the numbers of the hydrogen-requiring *C. concisus* isolated from human oral cavities and from faeces of patients with enteritis

(Vandamme *et al.*, 1989; On, 1994; Russell, 1995) following the application of improved isolation methods (le Roux & Lastovica, 1998). Although *C. concisus* had been linked to gingivitis and periodontitis (Moore *et al.*, 1987; Kamma *et al.*, 1994), its pathogenic role in oral cavity infections could not be established.

C. concisus strains were isolated from children with diarrhoea in different countries (Russell, 1995; Lindblom *et al.*, 1995; le Roux & Lastovica, 1998), and are known to be most common in some patient subgroups, mainly children under 24 months of age and immunocompromised patients (Engberg *et al.*, 2000; Lastovica & le Roux, 2000; Aabenhus *et al.*, 2002).

C. concisus is fastidious and characterized by low biochemical activity, which makes it hard to identify using conventional phenotypic techniques (Matsheka *et al.*, 2001). Furthermore, molecular typing methods such as DNA–DNA hybridization (Vandamme *et al.*, 1989), PFGE (Matsheka *et al.*, 2002), PCR (Bastyns *et al.*, 1995; Istivan *et al.*, 1998) and protein profiling (Aabenhus *et al.*, 2002) have shown it to comprise at least two

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Abbreviations: CHE, crude haemolytic extract; CHO, Chinese hamster ovary; HE, haemolytic extract; LPS, lipopolysaccharides; OMP, outer-membrane protein; OMPLA, outer-membrane phospholipase A; PC, phosphatidylcholine; PL, phospholipase.

molecular groups (genomospecies), which are phenotypically indistinguishable, but genetically divergent (Matsheka *et al.*, 2002).

Russell & Ward (1998) investigated *C. concisus* isolated at the Royal Children's Hospital, Melbourne and showed that some isolates have the ability to adhere and invade HEp2 cells, at a rate 4–100-fold higher than the controls of pathogenic and invasive *C. jejuni* and *C. coli* strains. Musmanno *et al.* (1998) found that a *C. concisus* strain isolated from stool samples in children with enteritis produced a cytotoxic-like effect on Chinese hamster ovary (CHO) cells similar to that shown by *C. jejuni* subsp. *doylei*, and induced intracytoplasmic vacuole formation similar to that caused by cytotoxic *Helicobacter pylori*, indicating the presence of different virulence factors in *C. concisus* clinical isolates and emphasizing the importance of investigating the presence of virulence determinants in this organism.

Phospholipases (PLs) constitute a very diverse subgroup of lipolytic enzymes that have the ability to hydrolyse one or more ester linkages in phospholipids with a phosphodiesterase as well as an acyl hydrolase activity. Phospholipases are diverse in the site of action on the phospholipid molecule, and their classification as phospholipases A₁, A₂, C and D is based on the site of cleavage of the attacked phospholipid (Waite, 1996).

As phospholipids and proteins represent the major chemical constituents of the host cell envelope, enzymes capable of hydrolysing these chemical classes, such as phospholipases, are likely to be involved in the membrane disruption processes that occur during host-cell invasion. Consequently, a wide variety of bacteria have evolved enzymes capable of hydrolysing phospholipids, which are key components of all eukaryotic bilayer membranes (Waite, 1996; Songer, 1997). An example is the PLA of Gram-negative bacteria, which is an integral membrane protein located in the outer membrane and is recognized as a virulence factor for a number of bacterial species (Merino *et al.*, 1999; Dekker, 2000; Matoba *et al.*, 2002).

Studies indicate that many bacterial outer-membrane or secreted phospholipases are haemolytic and could be responsible for tissue damage during infection (Songer, 1997). Such enzymes include PLA in pathogens such as *H. pylori* (Dorrell *et al.*, 1999; Tannaes *et al.*, 2001; Xerry & Owen, 2001), *Legionella* species (Flieger *et al.*, 2000), *C. coli* (Grant *et al.*, 1997), *Yersinia enterocolitica* (Schmiel *et al.*, 1998) and PLA₁ in *Aeromonas* species (Merino *et al.*, 1999).

We have previously reported (Istivan *et al.*, 1998) a haemolytic activity associated with lysis (haemolysis) of human and animal erythrocytes from 19 clinical strains of *C. concisus*, isolated from the faeces of children with gastroenteritis (Russell, 1995), and from the two reference strains ATCC 51561 and ATCC 51562. The prevalence of this β -haemolytic activity in ageing bacterial cultures suggested that it could be

a cell-associated or membrane-bound haemolysin(s), as has been previously detected in *C. jejuni* and *C. coli* (Wassenaar, 1997).

In the present study, we report the extraction and initial characterization of a PLA₂ membrane-bound haemolysin from *C. concisus* clinical strains, which were classified into two major molecular groups (genomospecies) based on their PCR product using primers designed to amplify the 23S rDNA region (Bastyns *et al.*, 1995; Istivan *et al.*, 1998). In addition to haemolytic action on mammalian erythrocytes, the haemolytic extract causes vacuolation and cytolysis of CHO cells. We also report the presence of sequence similarity between the PLA gene *pldA* of *C. coli* (Grant *et al.*, 1997) and a gene sequence present in all clinical isolates of *C. concisus* tested. The detection of PLA₂ activity in *C. concisus* strains suggests a possible pathogenic role for these bacteria in gastroenteritis and gingivitis.

METHODS

Bacterial strains and growth conditions. Nineteen *C. concisus* clinical isolates (RCH 3–RCH 21) were used in this study. Data on clinical histories for patients related to the isolates are in (Table 1). *C. concisus* strains were isolated from children with mild or severe diarrhoea at the Royal Children's Hospital, Melbourne, Australia, and were identified using standard phenotypic and biochemical tests (Russell, 1995). Two *C. concisus* reference strains, ATCC 51561, ATCC 51562, and the type strain ATCC 43264^T of the closely related species *Campylobacter mucosalis* (Vandamme *et al.*, 1989) were also used in this study. Haemolysin-positive *C. coli* strain 351 (a kind gift from N. Stern, USDA, GA, USA), *C. coli* NCTC 11366, *C. jejuni* strain 81116 and a *C. jejuni* isolate (RCH 2) from a child's blood culture (Russell & Ward, 1998) were also used in this study (Table 1). All *Campylobacter* strains were grown on Columbia blood agar base (Oxoid) supplemented with 5–7% (v/v) defibrinated horse blood, incubated at 37 °C for 2–4 days in anaerobic jars without anaerobic catalyst and flushed with a gas mixture consisting of 6% O₂, 8% CO₂, 6% H₂ and 80% balance of nitrogen. The cell pellet was collected and washed with PBS for the contact haemolysin assay, or with 10 mM Tris/HCl, pH 7.4, for the quantitative PLA₂ detection assay. Bacterial suspensions were prepared at 10⁹ c.f.u. ml⁻¹ in PBS or Tris/HCl and used when required. Stock cultures were preserved in tryptone milk (1% tryptone and 10% skimmed milk, w/v) at –70 °C.

Molecular characterization of *C. concisus* by PCR amplification.

C. concisus isolates were identified by PCR amplification of the 23S rDNA following the method described by Bastyns *et al.* (1995). This method was modified by using the two reverse primers (CON1 and CON2) independently rather than as a mixture, and was used to group the clinical isolates. Segments of the 23S rDNA were amplified using the following primer pairs: mixture A, forward primer MUC1 (5'-ATG AGTAGCGATAATTGGG-3') and reverse primer CON1 (5'-CAGTAT CGGCAATTCGCT-3'); mixture B, forward primer MUC1 and reverse primer CON2 (5'-GACAGTATCAAGGATTTACG-3') and mixture C with all three primers. RCH clinical isolates were grouped according to their PCR product with either of the reverse primer sets A or B, compared with the reference strains ATCC 51561 and ATCC 51562 (Istivan *et al.*, 1998).

All strains were subjected to a second PCR identification protocol as described by Matsheka *et al.* (2001) based on the DNA sequence of the 1.6 kb *Bgl*III–*Xba*I fragment of *C. concisus*. Forward (5'-AGCAGCATC TATATCACGTT-3') and reverse primers (5'-CCCCTTGATAGGC

Table 1. *Campylobacter* spp. strains used in this study

Age is shown in years, y, or months, m. D, Diarrhoea; G, gastroenteritis; V, vomiting (days of symptoms).

Strain no.	Source and clinical condition	Classification	Other strain designation
ATCC 51561	Faeces, 24 y, female	<i>C. concisus</i> ^{B*}	CCUG 20034
ATCC 51562	Faeces, 7 m, male, D	<i>C. concisus</i> ^A	CCUG 20700
ATCC 43264 [†]	Mucosa of porcine intestine	<i>C. mucosalis</i>	CCUG 10771 [†]
RCH 1	Faeces, human	<i>C. coli</i>	NCTC 11366
RCH 2	Blood, human	<i>C. jejuni</i>	RCH, Australia
RCH 3	Faeces, 1 y, male, D & V, (3)	<i>C. concisus</i> ^A	RCH, Australia
RCH 4	Faeces, 1 y, female, D & V, (5)	<i>C. concisus</i> ^A	RCH, Australia
RCH 5	Faeces, 5 m, female, D, (5)	<i>C. concisus</i> ^A	RCH, Australia
RCH 6	Faeces, 2 y, male, D, (3)	<i>C. concisus</i> ^B	RCH, Australia
RCH 7	Faeces, 16 m, male, G & V, (7)	<i>C. concisus</i> ^A	RCH, Australia
RCH 8	Faeces, 5 m, female, D & V, (2)	<i>C. concisus</i> ^A	RCH, Australia
RCH 9	Faeces, 2 y, male, D & fever, (7)	<i>C. concisus</i> ^A	RCH, Australia
RCH 10	Faeces, 3 m, male, D, (6)	<i>C. concisus</i> ^A	RCH, Australia
RCH 11	Faeces, 2 y, male, D, (4)	<i>C. concisus</i> ^B	RCH, Australia
RCH 12	Faeces, 30 m, male, D, (20)	<i>C. concisus</i> ^A	RCH, Australia
RCH 13	Faeces, 1 y, male, D, (2)	<i>C. concisus</i> ^A	RCH, Australia
RCH 14	Faeces, 2 y, female, G†, (5)	<i>C. concisus</i> ^B	RCH, Australia
RCH 15	Faeces, 5 m, female, G & V, (9)	<i>C. concisus</i> ^A	RCH, Australia
RCH 16	Faeces, 7 m, male, D & V, (2)	<i>C. concisus</i> ^B	RCH, Australia
RCH 17	Faeces, 13 m, male, D, (3)	<i>C. concisus</i> ^A	RCH, Australia
RCH 18	Faeces, 2 m, female, D & V, (7)	<i>C. concisus</i> ^A	RCH, Australia
RCH 19	Faeces, 2 y, female, D & V, (5)	<i>C. concisus</i> ^A	RCH, Australia
RCH 20	Faeces, 10 m, female, D & V, (14)	<i>C. concisus</i> ^A	RCH, Australia
RCH 21	Faeces, 16 m, male, D, (2)	<i>C. concisus</i> ^B	RCH, Australia
81116	Faeces, human	<i>C. jejuni</i>	UK, 1983
351	Chicken isolate	<i>C. coli</i>	USA

*Indicated as molecular group (genomospecies) A or B.

†More than one pathogen was detected.

GATAG-3') were used to amplify the 0.5 kb ORF3 region in *C. concisus* strains (Matsheka *et al.*, 2001). *C. jejuni* 81116, *C. coli* 351 and *C. mucosalis* ATCC 43264[†] strains were used as controls. In both PCR protocols, boiled bacterial-cell extractions were used as a DNA template for PCR. All PCR amplifications were carried out in triplicate to ensure the accuracy of this method.

Extraction of the membrane-bound haemolysin from bacterial cells.

Cell suspensions of 10¹⁰ c.f.u. ml⁻¹ of six *C. concisus* clinical isolates (RCH 3, RCH 5, RCH 6, RCH 7, RCH 11, RCH 15) and the two reference strains, ATCC 51561 and ATCC 51562, were sonicated. The sonicated cells were centrifuged at 15 000 g for 15 min at 4 °C. The cell debris was separated from the supernatant and tested for haemolytic activity using the contact haemolysin assay. To extract the membrane-bound haemolysin, cell debris from the previous preparation was incubated for 16–20 h at 37 °C in PBS with 0.05 % (v/v) Tween 20 and 1 mg lysozyme ml⁻¹ (Boehringer Mannheim). After incubation, the cell debris was separated by centrifugation at 6000 g for 15 min at 4 °C, and the supernatant was filtered through 0.22 µm filters, before assay for haemolytic and PLA₂ activities. This fraction, designated crude haemolysin extract (CHE), was then subjected to ultrafiltration, by passing through 100 and 30 kDa molecular mass cut-off YM filters (Amicon).

Retentates (R100, R30) and filtrates (F100, F30) from each ultrafiltration process were tested for haemolytic activity using the liquid haemolysis assay. R30 was designated haemolysin extract (HE) after testing for haemolytic activity. Protein concentrations in CHE, HE and bacterial cells were estimated using the modified Lowry method (Markwell *et al.*, 1987). All samples of the HE were tested for the presence of lipopolysaccharides (LPS) using the method described by Spiro (1966).

Haemolysin assays. The contact haemolysin assay was used to detect the haemolytic activity of *C. concisus* bacterial cells or cell pellets. Bacterial cell pellets were washed with sterile PBS and then resuspended in PBS to 1 × 10⁹ c.f.u. ml⁻¹. Equal volumes of the bacterial suspension and of 2 % (v/v) erythrocyte suspension were mixed in a sterile tube. The mixture was then centrifuged at 1000 g for 5 min to pellet the bacterial cells on top of blood erythrocytes. The tubes were then incubated at 37 °C under microaerophilic conditions for 18 h. A comparative negative control of sterile PBS and blood erythrocytes was also incubated under similar conditions. After incubation, the tubes were centrifuged at 1000 g for 5 min to pellet non-lysed cells and the OD₅₅₀ of the supernatants was measured. A positive control for complete haemolysis

(100%) was performed by replacing the same volume of bacterial suspension with distilled water in the test.

The liquid haemolysis assay was used to test for the haemolytic activity of HEs (with or without treatment with various agents). HEs (100 µl) were mixed with an equal volume of 2% (v/v) blood erythrocytes and incubated aerobically at 37 °C for 2–6 h, and then centrifuged at 1000 g for 5 min to pellet non-lysed cells and the OD₅₅₀ for the supernatants was measured. Negative controls of erythrocyte suspension mixed with equal volumes of PBS buffer with or without treatment with various agents were also incubated as indicated. Positive controls were performed by mixing equal volumes of 2% (v/v) lysed erythrocytes with PBS containing a similar concentration of the added compound. Rabbit erythrocytes, 2% (v/v) in PBS, were used in the haemolysis assays unless stated elsewhere. Both the contact and liquid haemolysis assays used in this study to quantify the haemolytic activity were repeated at least three times, using different samples for each bacterial strain. Moreover, each sample was tested in duplicate, and the mean of the OD₅₅₀ readings is reported.

PLA₂ assay. Quantitative PLA₂ activity of CHEs and R30 HE in Tris/HCl, pH 7.3, from *C. concisus* reference strains ATCC 51561 and 51562 and clinical isolates RCH 3, RCH 6, RCH 15 was detected using the secretory PLA₂ Correlate-Enzyme kit (Assay Designs), which also included a PLA₂ positive control, following the manufacturer's instructions. CHE samples from *C. jejuni* 81116, *C. coli* NCTC 11366 and *C. coli* 351 were also tested in this assay. All samples were tested for haemolytic activity, and lecithinase activity on egg-yolk agar plates; protein content was estimated by the modified Lowry method (Markwell *et al.*, 1987) before samples were tested using the kit. A strongly haemolytic crude extract of *Clostridium perfringens* with PLC but no PLA activity (a gift from Xenia Gastos, RMIT University, Melbourne) was used to confirm the sensitivity of the PLA₂ detection kit.

Proteolytic treatment. Bacterial cells cultivated for 48 h were washed and resuspended to 5×10^9 c.f.u. in PBS and the protein content estimated (Markwell *et al.*, 1987). Pronase solution (10 mg ml⁻¹; Boehringer Mannheim) was added to the cell suspensions at a ratio of 3:1 (w/w) cell protein/Pronase and the reaction mixture was incubated at 37 °C for 30 min. Pronase activity was then stopped by heating the reaction mixture for 30 min at 65 °C, and the Pronase-treated cells were washed in PBS to remove residual Pronase prior to assay for haemolytic activity. Controls used were: bacterial cell suspensions without Pronase treatment; suspensions without Pronase treatment heated at 65 °C for 30 min; and the negative and positive haemolysis controls. HE (containing 0.6 mg protein ml⁻¹) was also treated with Pronase at a ratio of 3:1 (w/w) HE/Pronase before testing for haemolytic activity and immunoblotting to characterize the nature of the haemolysis.

Preparation of outer-membrane proteins (OMPs), SDS-PAGE and immunoblot analysis. OMP profiles on SDS-PAGE were analysed to find a possible way of differentiating *C. concisus* isolates. OMPs were prepared from all *C. concisus* strains and the *C. mucosalis* type strain using 1% (w/v) Sarkosyl according to the method of Filip *et al.* (1973). Fifty micrograms of protein from each sample were loaded on SDS-PAGE gels and protein profiles were visualized after electrophoresis by Coomassie blue stain. For Western blotting, CHE, Pronase-treated CHE, HE and OMPs from *C. concisus* ATCC 51561 and from RCH 3 were solubilized in SDS-β-mercaptoethanol loading buffer, separated on a 12.5% polyacrylamide SDS gel (Laemmli, 1970) and transferred electrophoretically onto nitrocellulose membrane using a Trans-Blot apparatus (Bio-Rad Laboratories) as described by Towbin *et al.* (1979).

Polyclonal antiserum against whole bacterial cells of *C. concisus* RCH 6 was raised in female BALB/c mice by injecting 5×10^7 formalin-treated bacterial cells in PBS intraperitoneally; two booster injections were

given within 3 weeks before injecting 10^6 Sp2 myeloma cells intraperitoneally into the mice. The ascites fluid was drained from the injected mice from abdominal swellings after 2 weeks. The collected fluid was centrifuged at 6000 g for 10 min and the supernatant was used for immunoblotting.

Evaluation of the effect of physical and chemical factors on haemolytic activity. Bacterial cell suspensions in PBS and HE from *C. concisus* isolate RCH 3 were heated for 5, 10, 15, 20 and 30 min at different temperatures (45, 60, 75 and 100 °C) cooled on ice for 5 min and then used in the contact or liquid haemolysis assay to test for heat stability.

Calcium chloride and magnesium chloride were added separately to *C. concisus* cell suspensions or to CHE in final concentrations of 1, 10, 20, 40, 50, 100, 200 and 500 µM, while ferrous chloride was added at final concentrations of 1, 10, 20, 40 and 50 µM. The mixtures were incubated for 30 min at room temperature before erythrocytes were added to the haemolysis assay mixture. The effect of the calcium chelator EGTA on haemolytic activity was also tested by adding it to the assay at a final concentration of 1 mM with or without the presence of CaCl₂ under the same conditions. Negative controls of 2% erythrocyte suspension in PBS incubated with similar concentrations of CaCl₂, MgCl₂, FeCl₂ and EGTA in PBS were also tested in each assay to investigate the effect of these divalent cation concentrations on blood erythrocytes.

Inhibition of haemolysis with lipid substrates. Phospholipase inhibitors and protein kinase inhibitors resembling major classes of lipids found in biological membranes were tested in the liquid haemolysis assay to determine the nature of the *C. concisus* haemolysis. HE from RCH 3 was incubated for 30 min at room temperature with 20–60 µg ml⁻¹ final concentrations of each of the following compounds (prior to the addition of erythrocytes to the assay mixture): staurosporine (a protein kinase inhibitor), sphingomyelin, phosphatidylglycerol, phosphatidylcholine (a PLA inhibitor), *p*-nitrophenyl phosphoryl choline (a PLC inhibitor) and phosphatidylethanolamine. All compounds were obtained from Sigma. Inhibition of haemolytic activity was determined by comparison of results from samples with and without inhibitor. Negative controls contained 2% erythrocyte suspension in PBS incubated with the same concentrations of inhibitors.

Preparation of CHO cell cultures for evaluation of cytotoxic activity. The cytotoxic activity of HE (R30) and CHE was tested on CHO cells. Cells were routinely cultured in 25cm² tissue culture flasks containing 10–12 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) newborn calf serum (Trace Biosciences). The cultures were incubated at 37 °C with 5% CO₂. When used for cytotoxicity, confluent cells were washed with sterile PBS and harvested from culture flasks using trypsin/EDTA. The pellet was resuspended in 1.0 ml DMEM without serum. An aliquot of 0.1 ml of 1×10^5 CHO cells (estimated using a Neubauer counting chamber) was added to each well of a 96-well culture plate. Samples (100 µl) of serial twofold dilutions of CHE or HE (extracted from five different *C. concisus* strains) were then added separately to each well and incubated as above. The cells were tested for cytotoxic effect (visible rounding or cell damage) at 16, 24, 48 and 72 h. Filtrates from 30 kDa molecular mass cut-off filters (F30) were also tested on CHO cells for cytotoxic activity. CHE, HE and F30 samples were also tested for haemolytic activity before they were used in the cytotoxicity assay.

PCR amplification of *pldA* gene sequences from *C. concisus*. Two oligonucleotide primers, forward primer *FpldA* (5'-CCTCACCCCTTTA GAGAAACAA-3') and reverse primer *RpldA* (3'-GCTTCCTCCATAGC CATT-5'), were designed from the conserved region in the *C. coli* phospholipase A (*pldA*) gene (Grant *et al.*, 1997; Brok *et al.*, 1998) and used to amplify a 460 bp internal fragment of the homologous gene

from *C. concisus*. Genomic DNA prepared by the method of Maniatis *et al.* (1982) from *C. concisus* RCH clinical isolates, the two reference strains ATCC 51561 and ATCC 51562 and the control *C. coli* strain 351 were used as templates in this reaction. Due to the extracellular DNase activity found in *C. concisus* strains reported by Matsheka *et al.* (2002), the PCR amplification of the 460 bp internal fragment of *pldA* gene was repeated more than three times to obtain a clear PCR product from *C. concisus* genomic DNA.

RESULTS AND DISCUSSION

Molecular typing of *C. concisus* isolates

The detection of a 0.5 kb *C. concisus* species-specific PCR product (Matsheka *et al.*, 2001) in all RCH clinical isolates used in this study confirmed the strains to be *C. concisus*. No 0.5 kb PCR product was detected for isolates representing other *Campylobacter* species (data not shown).

By modifying the method for PCR amplification of the 23S rDNA region (Bastyns *et al.*, 1995) and using combinations of either MUC1-CON1 or MUC1-CON2, we were able to classify clinical *C. concisus* isolates from gastroenteritis cases in children into two genomospecies, where the majority of the isolates, 14 out of 19 (74%), and the reference strain ATCC 51562, were grouped in genomospecies A, while only five isolates (26%) and the reference strain ATCC 51561 were grouped in genomospecies B. Interestingly, the sequences of the two reverse primers, CON 1 and CON2, are significantly different, yet yield a similar-sized PCR fragment (306 bp), indicating a significant genomic difference between the two PCR groups. PCR molecular grouping and clinical histories for RCH isolates are summarized in Table 1.

Molecular identification and typing of *C. concisus* isolates has shown this species to be a complex species, that represents a taxonomic 'continuum', comprising several genomospecies (Matsheka *et al.*, 2002). Our results support the complex nature of *C. concisus* by demonstrating two molecular groups (genomospecies) within the Australian isolates involved in clinical cases of diarrhoea, with the majority of cases belonging to genomospecies A. However, the significance of these two genomospecies and their relatedness to pathogenesis is yet to be established.

Five different protein profiles were detected using SDS-PAGE analysis of OMP extracts for *C. concisus* strains, compared with the protein profile for the *C. mucosalis* reference strain (Fig. 1, lane 2). The majority of PCR group B strains showed an identical protein pattern (Fig. 1, lanes 8, 13, 18 and 23) except for the reference strain ATCC 51561 and strain RCH 14, which appear to be missing major polypeptide bands in the 43 kDa region (Fig. 1, lanes 3 and 16). However, PCR group A (genomospecies A) isolates demonstrated more diversity in their SDS-PAGE protein profile (Fig. 1, lanes 4–7, 9–12, 14, 15, 17, 19–22).

Recently, *C. concisus* isolates from immunocompromised patients with diarrhoea were differentiated into two groups depending on protein profiles of whole-cell lysates, with 85% of the isolates found to be from the same group (Aabenhus *et al.*, 2002). Immunodiffusion analysis (Vandamme *et al.*, 1989) and numerical analysis of AFLP profiles (On & Harrington, 2000) have also indicated the presence of two distinct genomospecies.

However, we have not yet aligned our two molecular grouping patterns for the Australian isolates with patterns generated by other researchers. Therefore, it remains unclear whether the genotype of the majority of *C. concisus* RCH strains assigned to PCR group A (genomospecies A) resembles that of the majority of *C. concisus* strains isolated from immunocompromised patients (Aabenhus *et al.*, 2002).

Haemolytic activity of bacterial cells and haemolysin extract

Different levels of haemolytic activity were detected in all *C. concisus* clinical isolates and reference strains using the contact haemolysin assay, even though clear haemolytic zones were not produced around colonies on blood agar cultures of some *C. concisus* and other *Campylobacter* species isolates. The pellet of sonicated *C. concisus* cells showed similar haemolytic activity to the intact cells. No haemolysis was detected when the supernatants, collected after sonication and centrifugation, were tested using the liquid assay, indicating a cell-bound haemolytic activity.

The CHE showed lower haemolytic activity compared with

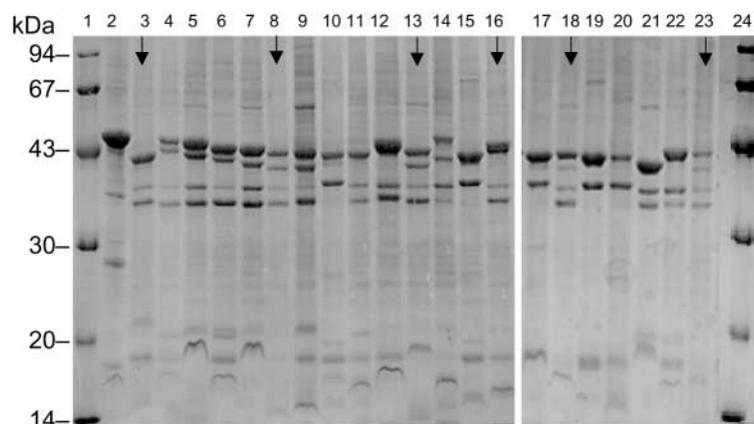


Fig. 1. SDS-PAGE of OMP profiles of *C. concisus* strains visualized by Coomassie blue stain. Lanes: 1 and 24, low molecular mass protein markers; 2, OMPs for *C. mucosalis* ATCC 43264^T; 3, OMPs for *C. concisus* ATCC 51561; 4, OMPs for *C. concisus* ATCC 51562; 5–23, OMPs for RCH *C. concisus* clinical isolates. Arrows indicate the six *C. concisus* strains assigned to genomospecies B, in lanes 3, 8, 13, 16, 18 and 23. The other 15 *C. concisus* strains were assigned to genomospecies A.

F100 filtrates and R30 retentates (HE), which completely haemolysed the erythrocytes after a short incubation period (1–3 h) when tested using the liquid haemolysin assay (100 % haemolysis). A similar pattern was observed when HEs from seven other *C. concisus* isolates representing both genomospecies were examined (data not shown).

The LPS test of the HE (Spiro, 1966) showed that all of the HE samples tested contained small amounts of LPS ($< 3 \mu\text{g ml}^{-1}$), compared with a positive control of LPS extracted from *C. jejuni* strain 81116 containing $500 \mu\text{g LPS ml}^{-1}$. Furthermore, *C. concisus* strains showed the presence of strong cell-associated haemolytic activity, with higher levels of haemolytic activity on human and rabbit erythrocytes when compared with *C. jejuni* and *C. coli* tested strains (Istivan *et al.*, 1998).

Haemolytic activity in all *C. concisus* isolates was membrane-bound. Sonication of cells did not release the haemolysin from cell debris and treatment with lysozyme and detergent was needed for effective haemolysin extraction, indicating the presence of a membrane-bound haemolysin (Deshpande *et al.*, 1997). Pronase-treated cell suspensions, CHEs and HEs showed negligible haemolytic activity in comparison with control untreated samples, when tested using the haemolysin assay, indicating that the haemolysin was proteinaceous in nature.

Quantitative detection of PLA₂ activity in HEs

Varying levels of PLA₂ activity, ranging from 25–135 units in 50 μl samples of CHE, were detected in *C. concisus* clinical isolates and reference strains, representing both genomospecies, when the secretory PLA₂ Correlate-Enzyme kit was used. PLA₂ activity for CHE from *C. jejuni* 81116, *C. coli* NCTC 11366 and *C. coli* 351 controls were 20, 26 and 55 U, respectively, in 50 μl samples (Fig. 2). Higher levels of PLA₂ activity were detected from HE (R30) of *C. concisus* strains,

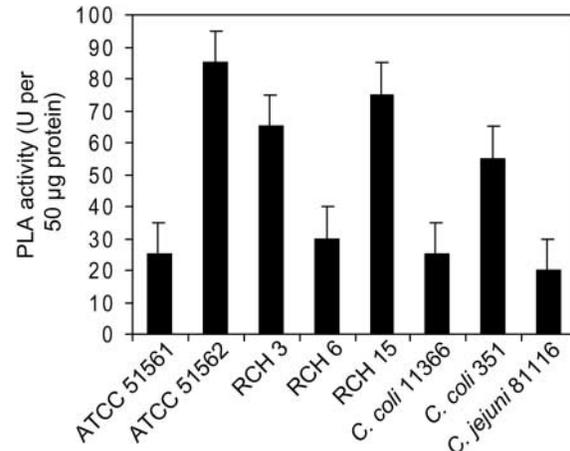


Fig. 2. PLA₂ activity in CHEs from *C. concisus* strains representing genomospecies A and B and from other *Campylobacter* strains. PLA₂ activity was detected using a commercial kit for detecting secretory PLA₂ activity in clinical fluid samples. PLA activity in 50 μl CHE samples protein was measured and plotted according to the standards provided in the kit.

with a range of 90–160 U per 50 μl sample. In HE samples from strain RCH 3, 1–2 U of PLA₂ activity ($\mu\text{g protein}^{-1}$)⁻¹ was detected, while CHE samples from the same strain had only 0.5–1 U PLA₂ activity ($\mu\text{g protein}^{-1}$)⁻¹ (Table 2).

To show that the phospholipase activity in *C. concisus* was not due to the presence of haemolytic PLC activity, CHE and HE samples and *C. concisus* bacterial cells were inoculated on egg yolk agar. No PLC (lecithinase) activity was detected on the medium compared with the control PLC sample from *Clostridium perfringens*. Furthermore, no PLA₂ activity was detected when an extract of PLC from *Clostridium perfringens* was tested using the secretory PLA₂ Correlate-Enzyme kit, confirming the specificity of the test kit.

Table 2. PLA₂ activity, haemolytic activity on 2 % rabbit erythrocytes and effect on CHO cells for different bacterial cell fractions of *C. concisus* RCH 3 strain

PLA₂ activity was quantitatively detected as units per 50 μl of sample (per μg protein) by a commercial kit. NT, Not tested.

Sample tested	PLA ₂ activity [U ($\mu\text{g protein}^{-1}$) ⁻¹]	Haemolysis (%) [*]	Effect on CHO cells
Intact bacterial cells	NT	60 ^a	NT
Sonicated cell pellet	NT	75 ^a	NT
Supernatant from sonicated cells	No activity	No haemolysis ^b	No effect
Crude haemolysin extract (CHE)	65 U [1 U ($\mu\text{g protein}^{-1}$) ⁻¹]	80 ^b	Vacuolation
R30 haemolysin extract (HE)	120 U [2 U ($\mu\text{g protein}^{-1}$) ⁻¹]	100 ^b	Cytolysis
Diluted haemolysin extract (HE)	58 U [1.4 U ($\mu\text{g protein}^{-1}$) ⁻¹]	100 ^b	Vacuolation
Pronase-treated haemolysin extract	NT	No haemolysis ^b	No effect
F 30	No activity	No haemolysis ^b	No effect

^{*}Haemolysis rate was detected by contact haemolysin assay (a) or liquid haemolysin assay (b) as a percentage of complete lysis of rabbit blood erythrocytes.

Immunoblot analysis of OMPs and HEs

A major immunoreactive band of around 34 kDa and a number of minor immunoreactive bands (39, 44, 67 kDa) from the OMPs, CHEs and HEs (Fig. 3, lanes 1, 2, 3, 4 and 6) were detected on the immunoblot using polyclonal anti-whole-cell *C. concisus* serum in standard samples but were absent after Pronase treatment of HE (Fig. 3, lane 5). This indicated the proteinaceous nature of this HE. The 34 kDa major immunoreactive band was present in the HE sample as well as a less intense 39 kDa band, but no higher molecular mass immunoreactive bands were detected in the HE sample.

The 34 kDa protein band was present in all haemolytic extracts with PLA₂ activity (data not shown) and could be considered a potential component of the haemolytic PLA protein molecule. This may account for the retentates (R30) retaining macromolecules with strong PLA₂ activity and haemolytic effect when tested on erythrocytes, while the filtrates (F30) did not show any effect (Table 2).

Protein molecules of approximately the same molecular mass with PLA activity have been reported in other enteric bacteria, such as PLA in *C. coli* with a predicted molecular mass of 35 kDa (Grant *et al.*, 1997), and in *Serratia* species, the predicted molecular mass for PLA₁ is 33.4 kDa (Song *et al.*, 1999), while the molecular mass for the outer-membrane phospholipase A (OMPLA) monomer in *Escherichia coli* is 31 kDa (de Geus *et al.*, 1983; Brok *et al.*, 1998). It could be suggested that the molecular mass of the enzyme monomer in *C. concisus* is approximately 34 kDa, by comparison with the major immunoreactive band on the blot, while one of the other minor bands (67 kDa) may represent the dimeric state of the enzyme.

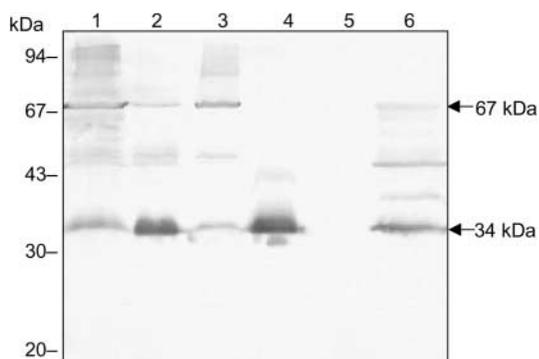


Fig. 3. Immunoblot for *C. concisus* extracts. Lanes: 1, ATCC 51561 OMPs; 2, ATCC 51562 OMPs; 3, RCH 3 OMPs; 4, HE from RCH3; 5, Pronase-treated HE from RCH 3; and 6, CHE from RCH 6. The blot was incubated with antiserum raised in mice injected with a suspension of whole bacterial cells from *C. concisus* RCH 6. The 34 and 67 kDa bands indicated are possible bands for PLA monomers and dimers, respectively.

The effect of different chemical and physical treatments on haemolytic activity

C. concisus haemolysin activity was not affected when cell suspensions were heated at 45, 60 and 75 °C for 10, 20 and 30 min, indicating thermal stability to 75 °C. The HE was more susceptible to heat treatment than were whole-cell suspensions, with a loss of 40 % of the total haemolysin activity after treatment at 75 °C for 10 min (data not shown). Haemolytic activity of cells and HE kept at –20 and –70 °C for several months showed no detectable change by both the contact and liquid assays, indicating that haemolysis was caused by a heat- and cold-stable factor.

Calcium ions had a notable effect on the haemolytic activity when added to the haemolysin assay mixture in varying concentrations compared with controls. When *C. concisus* bacterial suspensions or HEs were tested in the presence of Ca²⁺, there was a 5–15 % increase in haemolytic activity upon the addition of 1–10 µM CaCl₂. However, higher concentrations (20–500 µM) caused a gradual decrease in the haemolytic activity of HE (Fig. 4). Removing Ca²⁺ from the environment, by the addition of 1 mM EGTA as a calcium chelator, completely blocked the haemolytic activity, which could not be restored by the addition of extra Ca²⁺ (10–100 µM) to the assay (Fig. 4).

Ferrous ions also had a significant effect on haemolytic activity of *C. concisus* when tested using the contact haemolysin assay, with 10 µM FeCl₂ decreasing the activity to 40 % and 20 µM FeCl₂ to 20 % of the total haemolysis. Concentrations of 40 µM FeCl₂ or higher completely blocked the activity of this haemolysin (Fig. 4).

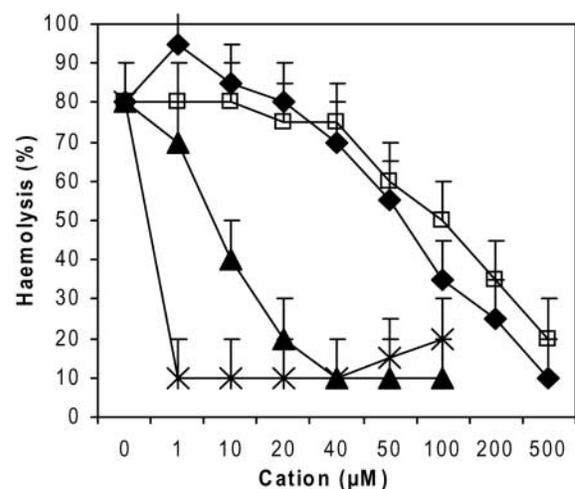


Fig. 4. Effect of divalent cations and the calcium chelator EGTA in the presence of CaCl₂ on haemolytic activity of *C. concisus*. Bacterial suspensions from *C. concisus* strain RCH 3 were tested using the contact haemolysin assay in the presence of 0–500 µM CaCl₂ (◆), 0–100 µM FeCl₂ (▲), 0–500 µM MgCl₂ (□) and 1–100 µM CaCl₂ with 1 mM EGTA (×). All samples were tested in triplicate.

The addition of Mg^{2+} had a weaker effect on haemolytic activity compared with the effect of Ca^{2+} and Fe^{2+} ions. Haemolytic activity was only reduced significantly when concentrations higher than $40 \mu M$ $MgCl_2$ were added to the contact haemolysis assay (Fig. 4). Moreover, the addition of Ca^{2+} , Mg^{2+} or Fe^{2+} ions to the haemolysin assay did not seem to have any effect on erythrocytes, compared with negative controls containing similar concentrations of these divalent cations.

Calcium ions are required for the catalytic activity of eukaryotic and prokaryotic PLA (Weinberg, 1985; Songer, 1997), such as the strictly calcium-dependent OMPLA in *E. coli* (Dekker, 2000). Recently, Ca^{2+} was found to play a catalytic and regulatory role in the formation and stabilization of the active dimeric state of OMPLA therefore preventing uncontrolled breakdown of the surrounding phospholipids in the outer membrane of the bacterial cell (Kingma & Egmond, 2002). It is likely that calcium also acts in a similar role in regulating the PLA₂ activity of *C. concisus*.

These results can best be explained by the presence of a heat-stable, calcium-dependent protein with haemolytic and PLA₂ activities in *C. concisus* similar to the haemolytic, calcium-dependent PLA in *C. coli* (Grant *et al.*, 1997) and to the calcium-dependent OMPLA of *E. coli*, which harbours PLA₁ and A₂ activities (Dekker, 2000).

The ability to acquire iron is essential for the bacteria to grow and, consequently, iron deficiency is a major factor involved in the induction of virulence determinants in pathogenic bacteria (Pickett *et al.*, 1992). Yields of bacterial enzymes and toxins in complex medium can be modulated by iron, such as lecithinase production in *Clostridium perfringens* and the α -haemolysin of *Staphylococcus aureus*, where the invasiveness of those pathogens is enhanced in strains that can release iron from host macromolecules (Weinberg, 1985). Ferric ions were found to have a significant effect on growth, OMP composition and siderophore synthesis in *C. jejuni* (Field *et al.*, 1986).

In this study, *C. concisus* haemolytic activity was reduced when ferric ions were added to the haemolysin bioassay, with a sudden decrease in the haemolytic activity of cells observed. In a previous study, we reported that growing *C. concisus* cells in an iron-deficient medium (using EDDA as an iron chelator) caused a detectable increase in haemolysis rates when these cells, and culture filtrates, were tested in the contact and liquid haemolysin assays, respectively (Istivan *et al.*, 1998). This was not unexpected, since a similar regulatory effect of calcium and iron on haemolytic activity was reported for *C. jejuni* and *C. coli* haemolysins (Hossain *et al.*, 1993; Grant *et al.*, 1997) and supports our theory that haemolytic activity is an important virulence factor in *C. concisus*.

Haemolytic activity in the presence of lipid substrates and phospholipase inhibitors

PC reduced haemolysis when used in the liquid haemolysin

assay with the HE. Concentrations of up to $60 \mu g$ PC ml^{-1} caused 60% reduction in haemolytic activity. Higher concentrations caused spontaneous lysis of the erythrocytes compared to PC-treated erythrocytes without HE, and therefore could not be tested for haemolysis. Other lipid substrates including NPPC, the PLC inhibitor, showed no or little effect on haemolysis (Fig. 5).

Lipase and phospholipase inhibitors have been used to study the nature, mode of action and structure of different phospholipases such as OMPLA in *E. coli* (Ubarretxena-Belandia *et al.*, 1999). In this study, PLC inhibitor and protein kinase inhibitor lipid substrates failed to show any inhibitory effect, whilst the *C. concisus* HE could be inhibited by the lipid substrate PC. Since the PLA inhibitors interact with a specific active site of the enzyme (Dekker, 2000), reactivity in this case implies a high degree of similarity between the active sites in the PLA₂ in *C. concisus* and bacterial PLA₂, which prefers PC to phosphatidylethanolamine as a substrate (Matoba *et al.*, 2002). PC is one of the main lipid components in the membranes of human and other mammalian erythrocytes, which explains the strong haemolytic effect of *C. concisus* haemolytic extract on human and rabbit erythrocytes (Istivan *et al.*, 1998). Membrane-bound phospholipases (haemolysins) active against the membranes of erythrocytes are widespread among enteropathogens including the PLA in *C. coli* (Grant *et al.*, 1997) and PLA₂ of *H. pylori* (Dorrell *et al.*, 1999).

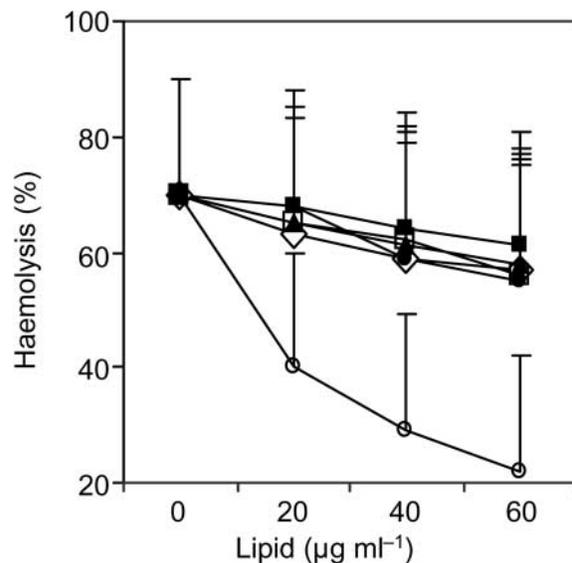


Fig. 5. Effect of lipids staurosporine (◇), sphingomyelin (■), phosphatidylglycerol (▲), phosphatidylcholine (○), *p*-nitrophenylphosphoryl choline (□) and phosphatidylethanolamine (●) on haemolytic activity of *C. concisus*. Lipids were added to HE from *C. concisus* strain RCH 3 at final concentrations of 20, 40 and $60 \mu g$ ml^{-1} . HEs were incubated with lipid substrates for 30 min before addition to the liquid haemolysin assay.

Effect of HEs on CHO cells

Addition of 100 µl diluted HE (containing 0.2 mg protein ml⁻¹) induced vacuolation of CHO cells after 16 h of incubation (Fig. 6a), while a similar volume of concentrated HE (containing 0.8 mg protein ml⁻¹) caused a strong cytolytic effect after 16 h of incubation (Fig. 6b). CHE had a weaker effect on the CHO cells at 16 h (Fig. 6c), with vacuolation noticed after 36 h of incubation. Both the cytolytic and the vacuolating effects were stable after 72 h of incubation. The haemolysin filtrates (F30) showed no visible effect on the normal growth of CHO cells even after 72 h of incubation (Fig. 6d).

The effect of haemolytic concentrates (HE) on CHO cells indicates the strong cytolytic (damaging) effect of the membrane-bound phospholipase haemolysin. Both diluted HE and CHE caused a distinct, stable, vacuolation effect on cells, with similar effects shown by HEs from both PCR groups (genospecies). All CHE and HE samples with vacuolating or cytolytic effect on CHO cells were haemolytic to erythrocytes when tested using the liquid haemolysin assay, while the F30 haemolysin filtrates had no cytotoxic effect on CHO cells and were not haemolytic to erythrocytes. PLA₂ activity, haemolytic activity and effect on CHO cells for different bacterial cell fractions are summarized in Table 2.

This intracytoplasmic vacuole-formation effect, similar to that caused by cytotoxic *H. pylori*, was also detected when *C. concisus* bacterial cells were incubated with Intestine 407 cells, while performing adhesion tests (Musmanno *et al.*, 1998). Haemolysins of *Serratia marcescens* and *Haemophilus ducreyi* have also been found to have a damaging effect on human epithelial cells (Hertle *et al.*, 1999; Wood *et al.*, 1999). PLA in *Legionella* species has been reported to cause destruction of lung surfaces and epithelial cells (Flieger *et al.*, 2000), and PLA of *H. pylori* is thought to have a role in colonization (Dorrell *et al.*, 1999). Further studies are required to determine whether the haemolysin isolated from *C. concisus*

produces a vacuolation effect on human epithelial cells similar to that produced by VacA toxin of *H. pylori* or VcVac from *Vibrio cholerae* (Massari *et al.*, 1998; Coelho *et al.*, 2000).

PCR analysis of PLA gene

Genomic DNA from two reference strains of *C. concisus* and other selected clinical isolates were tested by PCR using primers that amplified a 460 bp fragment of *C. coli* PLA (Grant *et al.*, 1997). PCR amplification of this region yielded similar-sized PCR products from genomic *C. concisus* DNA extracted from clinical isolate RCH 3, ATCC 51561, ATCC 51562 and *C. coli* USA 351 (Fig. 7, lanes 2, 3, 4 and 5, respectively).

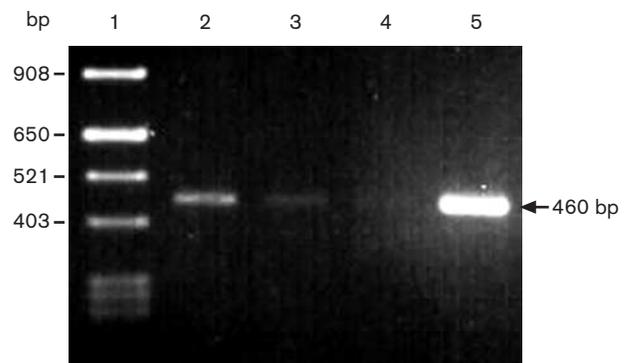


Fig. 7. PCR amplification of genomic DNA from *C. concisus* strains using primers for the conserved region of the *pldA* gene sequence of *C. coli*. The 460 bp PCR products were loaded on a 2% (w/v) agarose gel for electrophoresis and then stained with ethidium bromide and visualized on a UV trans-illuminator. Lanes: 1, low molecular mass DNA standards; 2, PCR product from *C. concisus* RCH 3; 3, PCR product from *C. concisus* strain ATCC 51561; 4, PCR product from *C. concisus* strain ATCC 51562; 5, PCR product from the *pldA* positive *C. coli* strain 351.

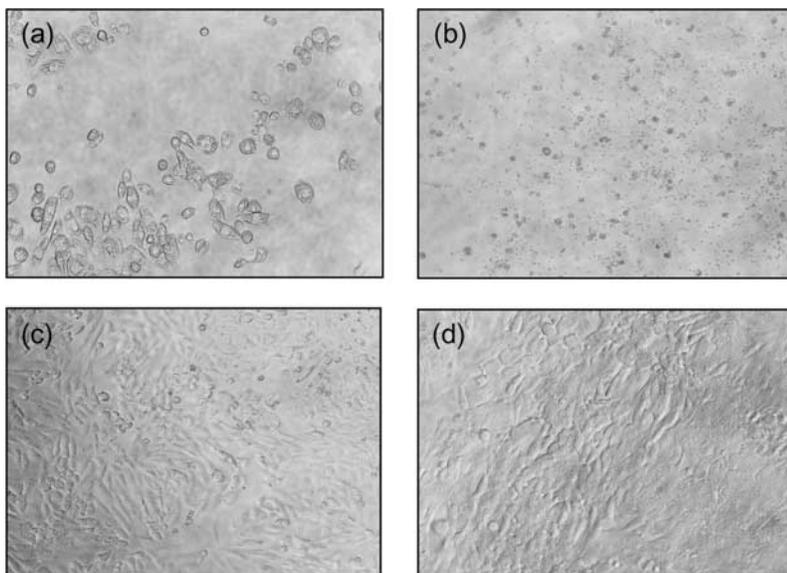


Fig. 6. Phase-contrast photomicrographs of *C. concisus* HE-induced vacuolation and cytolysis of CHO cells after 16 h. Confluent cells were cultured in DMEM supplemented with 0.1 ml PBS alone (d), or containing diluted HE (a), concentrated HE (b) and CHE (c). After incubation at 37 °C in a 5% CO₂ atmosphere for 16 h, cells were examined by phase-contrast microscopy. In (a), the vacuolating effect of diluted HE is visible, (b) demonstrates the cytolytic effect of concentrated HE, (c) demonstrates the weak effect of CHE and (d) shows normal CHO cells. Original magnification, ×200.

The genome of *C. concisus* strains demonstrate nucleotide sequence similarity to a 460 bp region of the *pldA* gene of *C. coli* PLA haemolysin. The existence of this sequence similarity suggests that a similar gene sequence for a haemolytic PLA enzyme exists in *C. concisus* strains assigned to both PCR groups (genomospecies). A *pldA* mutant of *C. coli* was previously shown to have marked reduction in haemolytic activity when compared with the wild-type strain, suggesting a role for *pldA* as a virulence determinant in campylobacters (Grant *et al.*, 1997), while a *C. jejuni pldA* mutant was reported to have an impaired ability for caecal colonization in chicken (Ziprin *et al.*, 2001). Similar research with a *H. pylori pldA* mutant has been reported, suggesting that the *H. pylori pldA* phospholipase has a role in colonization of the gastric mucosa and possible tissue damage after colonization (Dorrell *et al.*, 1999). Moreover, *pldA* gene has been related to colonization and persistence of *H. pylori* strains isolated from different geographical locations (Xerry & Owen, 2001).

Since haemolytic PLA₂ activity with a cytolytic effect is considered as a virulence factor in pathogenic bacteria (Songer, 1997) and is present in strains of the closely related species *C. jejuni* and *C. coli* (Brok *et al.*, 1998), the presence of similar activity in both genomospecies of *C. concisus* clinical isolates supports the possible virulence role of this phospholipase/haemolysin in the pathogenicity of *C. concisus*. Studies have been undertaken by our team to analyse the *pldA* gene in *C. concisus* as a potential virulence factor associated with tissue destruction that may be related to intestinal inflammation as a consequence of infection by *C. concisus*. Further molecular studies are needed to analyse the relatedness of *C. concisus* isolates assigned to the two genomospecies and their role in gastroenteritis and gingivitis.

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