Virulence determinants and production of extracellular enzymes in *Enterococcus* spp. from surface water sources

Lesego Gertrude Molale and Cornelius Carlos Bezuidenhout

**ABSTRACT**

Virulence factors in *Enterococcus* may be indicative of potential pathogenicity. The aim of this study was to determine the relationship between the presence of clinically relevant virulence genes, in *Enterococcus* sp. from environmental water, and their *in vitro* expression. One hundred and twenty-four *Enterococcus* isolates (seven species), from five surface water systems in the North West Province, South Africa, were screened for the presence of *asa1*, *cylA*, *esp*, *gelE* and *hyl* using polymerase chain reaction. The expression of *cylA*, *hyl* and *gelE* was determined by phenotypic assessments. Sixty-five percent of the isolates were positive for one virulence gene and 13% for two or more. Most frequently detected genes were *gelE* (32%) and *cylA* (28%). Enterococcal surface protein was absent in all isolates screened. The presence of virulence genes was correlated with their extracellular enzyme production. The results show that a large percentage of these environmental *Enterococcus* sp. possess virulence factors that could be expressed *in vitro*. This is a cause for concern and could have implications for individuals using this water for recreational and cultural purposes. Further investigation is required into the sources of these potential pathogenic *Enterococcus* isolates and measures to minimize their presence in water sources.

**Key words** | environmental enterococci, extracellular enzymes, surface water sources, virulence genes

**INTRODUCTION**

*Enterococcus* sp. are natural inhabitants of the intestinal flora of warm-blooded animals and humans (Lanthier et al. 2010). However, their presence in soil, surface waters, plants and vegetables has also been reported (Micallef et al. 2015). This ubiquitous nature of *Enterococcus* is a result of several intrinsic traits that enable their survival and adaption under harsh environments (Teixeira & Merquior 2013). These traits are attributed to their possession of easily transferable genes and highly effective gene transfer mechanisms such as conjugation and conjugal transposition (Eaton & Gasson 2001). According to Johnson (1994), the ability of *Enterococcus* to acquire and share elements encoding virulence traits has resulted in their increased importance as pathogens.

Virulence factors have been described as gene products that increase the ability of microorganisms to cause disease beyond the microorganism’s intrinsic properties (Mundy et al. 2000). Furthermore, they enhance the pathogenicity of a microorganism by permitting adhesion and invasion to a host’s tissue, abscess formation, translocation through epithelial cells, evading host immune responses and secretion of toxic products (Eaton & Gasson 2001). Studies of *Enterococcus* virulence mechanisms have become increasingly important because infections caused by this group of bacteria have, over the years, proven difficult to treat (Semedo et al. 2003). Various studies have reported the presence of virulence factors such as aggregation substance, gelatinase, cytolysin, enterococcal surface protein and hyaluronidase in *Enterococcus* sp. (Mundy et al. 2000; Eaton & Gasson 2001; Mannu et al. 2003). However, the majority of these studies have linked the subject of virulence to *E. faecalis* and *E. faecium* because of their significance in the clinical setting (Eaton & Gasson 2001; Ogier & Serror 2008). Nonetheless, reports of infection caused by other *Enterococcus* sp., such as *E. gallinarum*, *E. casseliflavus*, *E. mundtii*, and *E. hirae* have occasionally been documented (Mundy et al. 2000; Ahmed et al. 2012; Sidhu et al. 2014). The use of *Enterococcus* as indicators of faecal pollution is well documented and their presence in recreational and marine waters correlates with health risks (Cabelli & Dufour 1983). However, the relationship between the presence of virulent genes in...
Enterococcus spp. isolated from fresh water sources and whether these genes are expressed remains largely unknown (Sidhu et al. 2014).

The aim of this study was to determine the presence of five clinically relevant virulence genes (asa1, cylA, esp, hyl and gelE) in Enterococcus spp. isolated from environmental water sources in the North West Province, South Africa. In addition, the expression of three genes (hyl, cylA and gelE) was determined. This will aid in determining the potential pathogenicity of Enterococcus spp. isolated from environmental water sources and whether these may pose health risks to users.

MATERIALS AND METHODS

Bacterial strains

A total of 124 Enterococcus isolates were investigated in this study. All Enterococcus isolates were obtained from five surface water systems located in the North West Province, South Africa, during 2010 and 2011. These included four rivers (Harts, Vaal, Mooi, Schoonspruit rivers) and an inland lake (Baberspan). In addition, all isolates were identified to species level by phenotypic and molecular methods (Molale 2012). Enterococcus spp. analysed during this study included E. faecium, 30 isolates (24%); Enterococcus faecalis, 37 (30%); E. mundtii, 36 (29%); E. casseliflavus, 14 (11%); E. gallinarum, 5 (4%); E. hirae, 1 (0.8%); and E. sulfureus, 1 (0.8%).

Detection of virulence genes using PCR

Sequences of the virulence genes specific primers used in this study were from Vankerckhoven et al. (2004). The expected amplicon sizes of interest as well as all five oligonucleotide primer pairs (Applied Biosystems, UK) used to amplify the genes asa1, cylA, esp, gelE, and hyl are listed in Table 1. Identification of virulence genes for each isolate was performed by separate polymerase chain reaction (PCR). PCR amplifications were performed in a Techne Prime Elite thermocycler (Cambridge, UK), in 0.2 ml reaction tubes. Each PCR assay was performed in a total volume of 25 μl containing 1 μl bacterial DNA template (50–100 ng/μl), RNase/DNase free water (Fermentas Life Sciences, USA), 2× DreamTaq PCR Master Mix (0.05 U/μl Taq DNA polymerase in reaction buffer, 0.4 mM of each dNTP and 4 mM MgCl2), 0.2 μM of primers asa1 and gelE as well as 0.4 μM of primers cylA, esp, and hyl. Samples were amplified by denaturing at 95°C for 180 seconds, followed by 30 cycles of 95°C for 30 seconds, annealing at 56°C for 30 seconds and at 72°C for 60 seconds. This was followed by a final step of 72°C for 600 seconds. PCR products were confirmed by gel electrophoresis using a 1% (w/v) agarose gel in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) at 60 V for 90 minutes. A 100 bp molecular weight marker was used (O’GeneRulerTM 100 bp DNA ladder, Fermentas Life Sciences, USA). Agarose gel images were captured under UV light using a Bio-Rad ChemiDoc imaging system (Hercules, CA).

Sequence validation

The PCR products of representative isolates that were positive for the virulence genes asa1, cylA, gelE and hyl were purified as described by Li et al. (2010) followed by using the ZR DNA sequencing clean-up kit (Zymo Research, USA) according to the instructions of the manufacturer. Sequencing was performed and subsequent analysis was

<table>
<thead>
<tr>
<th>Virulence factor</th>
<th>Gene</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregation substance</td>
<td>asa1</td>
<td>ASA 11F – GCACGCTATTAGAAGACCTATGA ASA 12R – TAAGAAAGACATGCACAGGA</td>
<td>375</td>
</tr>
<tr>
<td>Cytolysin</td>
<td>cylA</td>
<td>CYT IF – ACTCCGGGAGATTGATAGCG CYT IFbR – GCTGCTAAAGCCTGCGGTT</td>
<td>688</td>
</tr>
<tr>
<td>Enterococcal surface protein</td>
<td>esp</td>
<td>ESP 14F – AGATTCTCGTTTGTCTTGG ESP 12R – AAATTGATCGTCCACCTGG</td>
<td>510</td>
</tr>
<tr>
<td>Gelatinase</td>
<td>gelE</td>
<td>GEL 11F – TATGACAATGTTTAGGGAATGG GEL 12R – AGATGGCACCAGAAATAATA</td>
<td>213</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>hyl</td>
<td>HYL n1F – ACAGAGAGCTGCGAGAATGG Hyl n2R – GACTGACGTCAAGTTCCTA</td>
<td>276</td>
</tr>
</tbody>
</table>
performed as described in Jordaan & Bezuidenhout (2013). Sequences were submitted to the GenBank database.

Phenotypic assays

Cytolysin

Cytolysin production was evaluated by observing β-haemolysis on blood agar plates. The haemolysis test was performed as described by Frobisher & Denny (1928). Briefly, purified overnight cultures were spot inoculated on 5% (v/v) sheep blood agar plates (National Health Laboratories, SA) and incubated at 37 °C for 24 hours. The β-haemolysis isolates caused complete cell lysis and were identified by a clear zone of hydrolysis around the colonies where inoculation occurred (Health Protection Agency 2008).

Gelatinase

The production of gelatinase was determined by the gelatin liquefaction protocol. Briefly, purified 18 hour streak plate cultures were stab inoculated into nutrient agar slants (Merck, Germany) supplemented with 12% gelatin (Oxoid, UK). After incubation at 28 °C for 7 days, the tubes were chilled for 30 min in cold conditions (5–10 °C). Cultures that remained liquefied were considered positive for gelatin hydrolysis (dela Cruz & Torres 2012).

Hyaluronidase

Hyaluronidase production was determined by spot inoculations on Brain Heart Infusion Broth (Merck, Germany) supplemented with 1.0 g of Noble agar (Difco Laboratories, USA) per 100 ml. Furthermore, an aqueous solution containing 5% (w/v) bovine albumin fraction V (Sigma Aldrich, USA) and 2 mg/ml of hyaluronic acid (Sigma Aldrich, USA) was filtered using minisart filters (0.20 μm; Separations, USA) and added to the media. A zone of clearing around the bacterial colonies was regarded a positive result (Hynes & Walton 2000).

Statistical analysis

Comparison of all Enterococcus spp. and the presence of virulent genes was achieved by converting data per enterococcus isolate into binary variables according to presence (1) and absence (0). These were ordered in Microsoft Office Excel 2007. All statistical analyses and graphs were performed using Statistica 12.0 (StatSoft, USA). The statistical association of Enterococcus spp. and virulence genes was determined using basic statistics correlation matrices and marked correlations were significant at $P < 0.05$ and $P < 0.001$.

RESULTS

PCR amplification and agarose gels were used to analyse the DNA of 124 Enterococcus isolates for the presence of the following virulence genes: asa1, cylA, esp, gelE and hyl. Figure 1 is the negative image of an agarose gel illustrating the PCR fragments of the four virulence genes identified in this study. The image also indicated the expected fragment lengths. No primer dimers or non-specific products were observed in any of the products. Furthermore, representative Enterococcus isolates positive for virulence genes asa1, cylA, gelE and hyl were sequenced and submitted to the GenBank database under accession numbers KT598460-KT98465 and KT724720-KT724721.

Occurrence of five virulence determinants in Enterococcus spp.

The Enterococcus spp. of interest comprised 30 Enterococcus faecium, 37 E. faecalis, 5 E. gallinarum, 14 E. casseliflavus, 36 E. mundtii, 1 E. hirae and 1 E. sulfurus isolate. Of the 124 Enterococcus spp. screened, 81 (65%) harboured at least

![Figure 1](https://iwaponline.com/wst/article-pdf/73/8/1817/461980/wst073081817.pdf)
one virulence gene, while 16 (13%) carried two or more virulence genes.

Prevalence levels of virulence genes per species are listed in Table 2. In addition, this table also illustrates the statistical association of the screened Enterococcus spp. with virulence genes. As depicted in Table 2, the most frequently detected virulence determinants were gelE and cylA. These genes accounted for 60% of the detected virulence determinants. Furthermore, virulence genes asa1 and hyl were also detected, though at lower levels. However, enteroococcal surface protein (esp) was not detected in any of the Enterococcus spp. screened.

A high prevalence of virulence genes was observed in E. faecalis isolates followed by E. faecium. The virulence gene cylA was present in six (86%) Enterococcus spp. and predominantly carried by E. gallinarum and E. casseliflavus spp. (Table 2). More so, the presence of cylA was statistically significant in four of the six species it was detected in (Table 2). The virulence genes asa1, gelE and hyl were each detected in four (57%) Enterococcus spp. (Table 2). Furthermore, the presence of asa1 was significantly higher in E. mundtii isolates compared to the others (Table 2), whereas, the presence of gelE was frequently and significantly detected in E. faecalis spp. Lastly, a statistically significant high frequency of the hyl gene was observed in E. gallinarum spp.

Production of extracellular enzymes

The production of three extracellular enzymes (haemolysin, gelatinase and hyaluronidase) was determined in the Enterococcus isolates. As depicted in Table 2, the most frequently detected extracellular enzymes were β-haemolysis and gelatinase. Haemolysin activity was observed in four Enterococcus spp., representing 57% of the isolates. However, only two of the Enterococcus spp., representing 29% of the isolates, produced gelatinase and hyaluronidase (Table 2). Furthermore, haemolysin activity was predominantly observed in E. gallinarum spp.; however, this characteristic also showed a statistically significant association with E. casseliflavus and E. mundtii spp. (Table 2). On the other hand, gelatinase production was statistically significant amongst E. faecalis as compared to the other Enterococcus spp.

Table 2 | Observed presence of virulence determinants and the production of extracellular enzymes in seven Enterococcus spp.

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates (N)</th>
<th>Virulence genes</th>
<th>Extracellular enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. faecium</td>
<td>30</td>
<td>asa1</td>
<td>β-Haemolysis (n (%))</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cylA</td>
<td>Gelatinase (n (%))</td>
</tr>
<tr>
<td></td>
<td></td>
<td>esp</td>
<td>Hyaluronidase (n (%))</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>37</td>
<td>3 (10.0)</td>
<td>2 (6.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 (40.0)*</td>
<td>4 (15.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1 (3.3)</td>
</tr>
<tr>
<td>E. gallinarum</td>
<td>5</td>
<td>4 (80.0)**</td>
<td>2 (5.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>5 (14.0)**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1 (3.0)</td>
</tr>
<tr>
<td>E. casseliflavus</td>
<td>14</td>
<td>1 (7.1)</td>
<td>4 (80.0)**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11 (79.0)**</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E. mundtii</td>
<td>36</td>
<td>8 (22.0)**</td>
<td>6 (43.0)**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 (11.0)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E. hirae</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E. sulfureus</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>124</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>9</td>
<td>2</td>
</tr>
</tbody>
</table>

Percentage (%) was determined as a function of the number of isolates of the specific Enterococcus spp. thus \( \frac{n}{N} \times 100 \); Correlation significance: ** \( P < 0.001 \), * \( P < 0.05 \).

DISCUSSION

In spite of major improvements in water and wastewater treatments as well as recommendations made by regulatory bodies, several studies in South Africa have reported the poor operational state of wastewater treatment plants, resulting in pollution of receiving water bodies (Momba et al. 2009;
Additionally, studies reporting the discharge of wastewaters containing significant pathogenic microorganisms into receiving water bodies have indicated that this is a worldwide problem (Okoh et al. 2007).

Surface waters receiving wastewater effluents are used as source water sources for drinking water production. Wastewater effluents can be reused for irrigation, and indirect or direct potable reuse purposes (Weinberg et al. 2004; Sidhu et al. 2014). Exposure to environmental surface water systems contaminated by various sources of pollution could lead to transmission of potentially pathogenic Enterococcus spp., resistant to multiple antibiotics, which have previously been isolated from surface water systems of the North West Province (Molale 2012).

In the present study, 124 Enterococcus spp. isolated from environmental surface water systems in the North West Province, South Africa, were screened for the presence of five clinically relevant virulence factors (asa1, cylA, esp, gelE and hyl). The high incidence of gelE genes in Enterococcus isolates screened in this study is in accordance with several other studies (Eaton & Gasson 2001; Doğru et al. 2010; Di Cesare et al. 2014). Furthermore, the observation of gelE positive isolates being significantly more common in E. faecalis isolates screened in this study is in accordance with the findings of similar other studies (Ahmed et al. 2012; Hammad et al. 2014; Sidhu et al. 2014). In the case of the present study gelE detection was not confined to E. faecalis. It was also associated with several Enterococcus spp. This is supported by several studies that reported the presence of gelE genes also in E. faecium, E. mundtii, E. durans and E. casseliflavus (Macedo et al. 2011; Sidhu et al. 2014).

The high prevalence of cylA positive Enterococcus spp. identified in this study is in contrast to numerous studies where no or very few cylA genes were detected in Enterococcus isolates screened (Lanthier et al. 2010; Ahmed et al. 2012; Iweriebor et al. 2013). However, the presence of the cylA gene amongst six of the seven Enterococcus spp. that were screened in the present study confirms an observation by Semedo et al. (2005) that cylA genes are widely distributed throughout the Enterococcus genus. A study by Ahmad et al. (2014) predicted that cylA positive isolates from river water systems have urban flow as the potential source. They advocated the use of this gene as a marker for human faecal pollution. Taking this into account suggests that isolates in the present study could have originated from human faecal matter. This is an aspect that should be further investigated.

Similar to the findings of Sidhu et al. (2014) the plasmid-bound asa1 gene was more frequently prevalent in E. faecalis and E. mundtii isolates. In the present study this gene was also detected in E. faecium and E. casseliflavus. These findings support suggestions of previous studies in that the asa1 gene has a significant distribution amongst Enterococcus spp. isolated from fresh water sources (Macedo et al. 2011; Sidhu et al. 2014). Since this gene is located on a plasmid (Sidhu et al. 2014) it may explain the wide distribution thereof among Enterococcus spp. from water systems, as plasmids provide a means for the dissemination of such genes among aquatic bacteria.

The hyl gene was detected among various Enterococcus species (Table 2). This is in accordance with the findings of Gonçalves et al. (2011) and Trivedi et al. (2011). The latter authors showed that E. faecalis, E. casseliflavus, E. mundtii, E. durans and E. gallinarum could all be
associated with carrying this gene. However, the low prevalence of the hyl gene among environmental isolates should not be regarded as uncommon. Even in the case of clinically relevant isolates, Rice et al. (2003) reported lower prevalence levels of hyl positive Enterococcus spp. from community-acquired isolates when compared to isolates from a hospital environment. In the present study, none of the 36 E. mundtii isolates tested were positive for the hyl gene. This may not be surprising as recent studies of Iweriebor et al. (2015a, 2015b) demonstrated that various Enterococcus spp. isolated from two different environments in South Africa were also negative for the hyl gene.

The esp gene was not detected amongst any of the various environmental Enterococcus isolates. This gene is associated with a 150-kb putative pathogenicity island (PAI) usually found in clinical isolates (Leavis et al. 2004) contributing to urinary tract infections (Shankar et al. 2001). In various previous studies, esp positive Enterococcus spp. were isolated from human sewage, fecal samples, fresh water, wastewater and septic samples (Scott et al. 2005; Sidhu et al. 2014; Iweriebor et al. 2015b). These were all associated with severe human faecal pollution. Not finding the esp gene amongst any of the 124 Enterococcus spp. suggests that (i) the isolates were not from fresh human faecal pollution events, or (ii) that the esp gene PAI was absent or (iii) that it was incomplete. This is in agreement with the findings of Shankar et al. (2006) who illustrated that esp negative isolates can contain fragments of the PAI. Furthermore, Shankar et al. (2006) demonstrated variation in the genetic content of the PAI. Thus, the absence of the esp gene could imply that the PAI, if present, was incomplete.

Presence of virulence genes and production of extracellular enzymes

Forty percent of the cylA gene carrying isolates produced β-haemolysis on blood agar. The low β-haemolysis positive isolates from this study are similar to results of Sidhu et al. (2014). The latter authors suggested the low spread of β-haemolysis activity in Enterococcus spp. isolated from water sources. Furthermore, it has been demonstrated that cytolysin expression requires the products of eight genes on the cylL-L8ABM operon (Semedo et al. 2005; Poeta et al. 2008). It is thus possible that isolates incapable of producing β-haemolysis in this study did not harbour all genes of the cylL-L8ABM operon. However, according to Franz et al. (1999), the absence of haemolytic activity in Enterococcus isolates does not mean they are not virulent. This is in agreement with a study by Macovei & Zurek (2006) which showed a 100% correlation between cylA and β-haemolysis on human blood as opposed to cattle blood. In the present study, sheep blood agar plates were used and this could have played a role in the low levels of β-haemolysis production.

Similar to the findings of Creti et al. (2004), the presence of gelE positive Enterococcus spp. in this study was not correlated with the production of gelatinase. Eaton & Gasson (2001) suggest that this minimal expression of the gelE gene is due to existence of silent gelE genes. Furthermore, it has been illustrated that the presence of the gelE gene is not enough for gelatinase production if the 23.9-kb region including most of the fsr locus, a positive gelE expression regulator, is absent (Bourgogne et al. 2006). Thus in the present study, the expression regulator might have been absent or repressed among the gelatinase negative isolates.

Hyaluronidase activity was observed among 22% of the hyl gene carrying isolates. According to Eaton & Gasson (2001) the absence of phenotypic activity in virulence gene carrying isolates may be a result of down regulation of gene expression or may be indicative of an inactive gene product. These two processes could have been responsible for the low hyaluronidase activity among the isolates from the present study that carry the hyl gene.

CONCLUSION

Water pollution is amongst the key environmental problems experienced in South Africa. Municipal wastewater treatment plants not working efficiently or not fully operational are amongst the many sources responsible for polluting environmental water systems by spilling poor quality effluents into receiving surface water bodies. This results in the degradation of the country’s surface water systems, which support various social needs. In this study, PCR was used to screen for the presence of five clinically relevant virulence factors among seven species from the genus Enterococcus isolated from surface water of the North West Province of South Africa. Four of these factors were detected among the various species and showed statistical significant associations with species. The isolates were also tested to determine if three of the genes (cylA, gelE and hyl) are expressed into functional units that could be associated with virulence. The number of isolates that were positive for these three virulence factors were much lower than those that produced the functional gene products causing the phenotypic characteristics. There could be various reasons for the non-expression of the phenotypic trait, among those, repression of the gene or absence of critical
factors that may be present/provided under in vivo conditions. Finding these genes amongst a large number of Enterococcus spp. isolated from surface water may thus constitute a human infection risk. On the other hand, if these genes are not directly involved in causing infections, their presence in surface water should be considered a cause for concern. If these genes are associated with mobile elements, such as plasmids, in environmental populations, environmental Enterococcus spp. could act as reservoirs and sources of such virulence genes for dissemination. Dissemination of such carriers of virulence genes in environmental surface water sources that are used for various agricultural, religious, cultural and recreational activities expose those individuals and animals to serious health risks. It is thus recommended that recreational and agricultural water be regularly tested for the presence of Enterococcus and that appropriate intervention methods be put in place if the water does not comply with specified standards.

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