Occurrence, antibiogram and vancomycin resistance of generic enterococci in horses in Nigeria

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ABSTRACT

This study was conducted to isolate generic enterococci from horses in Nigeria, and to determine the antibiotic resistance profile and phenotypic vancomycin (VAN) resistance of the isolates. Non-duplicate rectal swabs were collected from 200, randomly-selected apparently-healthy horses. Isolation of enterococci was done using Slanetz and Bartley enterococcal selective medium. Resistance of 30 non-repetitive/duplicate isolates was determined using disc diffusion method. VAN resistance was assessed by high-level disc diffusion and agar screening methods while high-level aminoglycosides (gentamicin and streptomycin) resistance was determined by agar screening method. Out of 200 samples, 197 (97%) gave positive growth. From these, 210 enterococcal isolates comprised of 13 (6.2%) haemolytic and 197 (93.8%) non-haemolytic strains, were obtained. Of the 30 isolates, 11 (36.7%) were resistant to chloramphenicol, 24 (80%) to erythromycin, 15 (50%) to tetracycline, 27 (90%) to rifampicin and 7 (26.7%) to VAN. Of the 7 VAN-resistant isolates, 3 (42.9%) were haemolytic while 4 (57.1%) were non-haemolytic. An isolate was susceptible to all the antibiotics tested. Among 29 resistant isolates, 18 (62.1%), including 6 (85.7%) of the 7 VAN-resistant strains, exhibited resistance to at least 3 classes of antibiotics. This study showed that horses in Nigeria are potential reservoirs and disseminators of multidrug- and VAN-resistant enterococci.

Keywords: enterococci, antibiogram, vancomycin-resistance, equine

Introduction

Enterococci are natural inhabitants of the intestinal tract in human and animals, including horses [28, 36]. Because of their ubiquity in faeces and adaptive capacity, they are found in various ecological niches such as soil, water (serving as indicator of faecal contamination) and food (serving as indicator for sanitary quality) [16, 36]. Nowadays, these organisms are important causes of various invasive and non-invasive infections in humans and animals; and they are recognized as one of the leading causes of hospital- and community-acquired infections worldwide [16, 19, 41]. There is increased interest in antibiotic-resistant enterococci (ABRE), especially the multidrug- and vancomycin (VAN)-resistant strains, because these organisms possess intrinsic resistance mechanisms against many classes of antibiotic (such as lincosamides, streptogramins, polypeptides, β-lactams and low-level aminoglycosides and VAN) and they easily acquire and transfer diverse resistance genes to clinically-relevant organisms (especially staphylococci), thereby limiting therapeutic options in treating enterococcal infections [16, 36]. The use of glycopeptides (VAN and avoparcin) stimulates selection against high-level VAN resistance in enterococci [8, 36]. VAN is critically-important in the management of severe infections in human and animals worldwide, thus VAN-resistant enterococci (VRE) was recently classified as “high priority pathogens” that pose threat to human and animal health against which new treatment strategies and more researches documenting their occurrence in different reservoirs, are urgently needed [1, 42].

The population of horses in Nigeria is estimated at 200,000-240,000 with most of these being raised in the northern region of the country [32, 37]. These horses are intensively managed and extensively used for various purposes including ceremonies (dubar festival in the northern region and funerals in the southern part), sports (polo games), research, security (police and military mountain troops) and as source of...
animal protein (in the southern region) by slaughtering spent (used) horses transported from the northern region [4, 5]. Because the use of antibiotics (including critically-important ones like VAN) in Nigeria is uncontrolled, veterinarians and non-professionals without veterinary supervision, may be managing infected horses with various types of antibiotics, including VAN, to ensure therapeutic success owing that horses are prized animals [4]. Thus, these horses may harbour ABR-/VRE. These organisms harboured by these horses, could be discharged (in faeces) into the environment, thereby serving as reservoirs and disseminators of genes encoding antibiotic/VAN resistance to other pathogenic bacteria by horizontal gene transfer thereby complicating infections and jeopardizing therapy [19].

Evidences support zoonotic transmission of ABR-/VRE from animals to humans [1, 34, 40]. In rural northern Nigeria, livestock, horses and humans are often in close contact, thus a putative risk for exchange of ABR-/VRE between these hosts [4]. Individuals in direct contact with these horses (such as veterinarians, jockeys and handlers of these horses) and those in indirect contact via contaminated environment (children/adults playing in fields contaminated with horse faeces) and food chain are also potential risk for transmission of these organisms [41]. Enterococci in faeces of horses at slaughter in Nigeria can easily contaminate the slaughterhouse environment, workers, meat and associated products because of poor hygienic practices in the slaughterhouses [39, 41]. These factors can consequently result in fast spread of ABR-/VRE in human and animal population in Nigeria.

There are few reports [17, 33, 38] on isolation of ABR-/VRE from clinical samples of horses. Studies assessing healthy horses as potential reservoirs of ABR-/VRE are scanty and included reports in America [35], India [33], Belgium [14], Italy [13], Portugal [27, 28] and Korea [19]. In Nigeria, poultry [2, 6, 30, 31], pigs [2, 7] and cattle [3, 31] have been screened as potential reservoirs of ABR-/VRE. The potential of horses in Nigeria as reservoirs of ABR-/VRE has remained uninvestigated. Phenotypic susceptibility profile of ABRE is important for guiding empiric treatment of infection associated with these organisms in horses. The objective of this study, therefore, was to determine the occurrence of enterococci in horses at slaughter in the Obollo-Afor market in Udenu Local Government Area (L. G. A.), Enugu State Southeast Nigeria and assess the antibiotic susceptibility profile and phenotypic VAN resistance of the isolates.

Materials and methods

STUDY AREA

Obollo-Afor is a town in Udenu L. G. A. of Enugu State, southeastern Nigeria. It is geographically located at coordinates 6.9153° N and 7.5139° E. Obollo-Afor market is a major horse-selling and slaughtering point (average of 20 horses are killed daily) in Enugu State [4].

SAMPLING

Horses presented for slaughter at the Obollo-Afor market/slaughter slab between March and June, 2018 were sampled. Two hundred horses were selected using random sampling technique. Prior to slaughter, non-duplicate rectal swab was collected from each of the horse using a sterile swab stick. The samples were transported aseptically in ice packs and processed the day of collection in the Veterinary Microbiology Laboratory, Department of Veterinary Pathology and Microbiology, University of Nigeria.

ISOLATION AND GENERIC IDENTIFICATION OF ENTEROCOCCI FROM HORSES

The swabs were inoculated into tryptone soy broth with 6.5% salt and incubated at 37°C for 48 hours in ambient air. A loopful of the broth cultures was sub-cultured onto Slanetz and Bartley agar and incubated at 37°C for 48 hours for selective isolation of enterococci. Morphotypes (pinkish, reddish or maroon-coloured tiny colonies) of putative enterococci were noted and recorded appropriately. One colony per morphotype per sample was picked and purified by sub-culturing on nutrient agar plates and incubated at 37°C for 48 hours. Pure cultures of the isolates were then inoculated onto nutrient agar slants, incubated at 37°C for 48 hours and stored in a refrigerator at 4°C as stock cultures until needed for further analysis. Phenotypic characterization of the isolates to generic level was done by subjecting them to various tests such as Gram staining, catalase, bile esculin, haemolysis, growth in 6.5% salt, growth at 45°C, and tolerance of 60°C for 30 minutes, following standard methods [25].

DETERMINATION OF ANTIBIOTIC SENSITIVITY TESTING PROFILE AND PHENOTYPIC VAN RESISTANCE OF THE ISOLATES

Antibacterial susceptibility profiles of 30 non-repetitive/duplicate (1 isolate per horse) enterococcal isolates were determined by the disc diffusion method [10], using 7 antibacterial agents in 6 classes: ansamycins – rifampicin (RIF; 5µg), tetracycline (TET; 30 µg), phenicols - chloramphenicol (CHL; 30 µg), macrolides – erythromycin (ERY; 15 µg) and glycopeptides – VAN (30 µg). High level aminoglycosides (high level gentamicin [Hlg] and high level streptomycin [Hls]) resistance was determined by agar screening method using gentamicin (GEN; 500 µg/mL) and streptomycin (STR; 2000 µg/mL) [11]. Enterococcus faecalis ATCC 29212 (American Type Culture Collection, USA) was used as a reference strain. Results of the susceptibility testing were interpreted according to the CLSI (2018b) [11] criteria for enterococcal isolates. The multiple antibiotic resistance index (MARI) of the isolates was determined using the formula a/b where 'a' is the number of antibiotics to which an isolate was resistant and 'b' the number of antibiotics the isolate was exposed [22]. Mean MARI was calculated as the ratio of total MARI and total number of resistant isolates. An

isolate resistant to ≥ 3 classes of antibiotics was considered multidrug-resistant (MDR) [24].

**ASSESSMENT OF PHENOTYPIC VANCOMYCIN RESISTANCE**

Phenotypic VAN resistance of the 30 non-repetitive/duplicate enterococcal isolates was determined using high level (30 µg) VAN disc and agar screening (6µg/mL of VAN) methods [10]. *Enterococcus faecalis* ATCC 29212 was used as reference strain. Results of the susceptibility testing were interpreted according to the CLSI (2018b) [11] criteria for enterococcal isolates.

**DATA ANALYSIS**

The frequencies of occurrence of enterococci and resistance of the isolates to antibiotics were entered into Microsoft Excel version 2010 (Microsoft Cooperation Redmond, USA) and their percentages calculated. The 95% confidence interval (CI) of the resistance frequencies was also calculated using SPSS v 15 software.

### Results

**OCCURRENCE OF GENERIC ENTEROCOCCI IN HORSES**

Out of 200 rectal swabs cultured, 197 (97%) gave positive growth of enterococci. From these, 210 isolates comprised of 13 (6.2%) haemolytic and 197 (93.8%) non-haemolytic strains were obtained.

**ANTIBIOGRAM OF GENERIC ENTEROCOCCAL ISOLATES FROM HORSES**

Of 30 isolates, 11 (36.7%, 95% CI 19.5-53.9) were resistant to CHL, 24 (80%, 95% CI 65.7-94.3) to ERY, 15 (50%, 95% CI 32.1-67.9) to TET, 27 (90%, 95% CI 79.3-100.7) to RIF and 7 (26.7%, 95% CI 8.17-38.43) to VAN (Table 1). None of the isolate was resistant to HLS and HLG. An isolate was susceptible to all the antibiotics tested.

Among 29 resistant isolates, 4 (13.8%) were resistant to 1 class of antibiotic, 7 (24.1%) to 2 classes while 18 (62.1%), including 6 of the 7 VAN-resistant strains, were resistant to 3 or more classes of antibiotic (Table 2). Ten multiple resistance

<table>
<thead>
<tr>
<th>Antibiotic (concentration)</th>
<th>Number (Percentage) of isolate</th>
<th>n = 30</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Susceptible</td>
<td>Intermediately-susceptible</td>
</tr>
<tr>
<td>Vancomycin (30 µg)</td>
<td>8 (26.7)</td>
<td>15 (50)</td>
</tr>
<tr>
<td>Chloramphenicol (30 µg)</td>
<td>12 (40)</td>
<td>7 (23.3)</td>
</tr>
<tr>
<td>Erythromycin (15 µg)</td>
<td>1 (3.3)</td>
<td>5 (16.7)</td>
</tr>
<tr>
<td>Tetracycline (30 µg)</td>
<td>8 (26.7)</td>
<td>7 (23.3)</td>
</tr>
<tr>
<td>Rifampicin (5 µg)</td>
<td>3 (10)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Gentamicin (2000 µg/mL)</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Streptomycin (500 µg/mL)</td>
<td>30 (100)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

**Table I**: Antibiotic susceptibility profile of generic enterococcal isolates from horses

<table>
<thead>
<tr>
<th>Number of antibiotic class</th>
<th>Resistance pattern (Number of isolates)</th>
<th>Total number of isolates (Percentage)</th>
<th>MARI (Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CHL (1)</td>
<td>4 (13.8)</td>
<td>0.14 (0.56)</td>
</tr>
<tr>
<td></td>
<td>RIF (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERY-RIF (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>VAN-RIF (1)</td>
<td>7 (24.1)</td>
<td>0.29 (2.03)</td>
</tr>
<tr>
<td></td>
<td>TET-RIF (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERY-TET-RIF (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CHL-ERY-RIF (2)</td>
<td></td>
<td>0.43 (3.01)</td>
</tr>
<tr>
<td></td>
<td>CHL-ERY-TET (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CHL-ERY-TET-RIF (4)</td>
<td>18 (62.1)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>VAN-CHL-ERY-RIF (1)</td>
<td></td>
<td>0.57 (3.99)</td>
</tr>
<tr>
<td></td>
<td>VAN-ERY-TET-RIF (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>VAN-CHL-ERY-TET-RIF (3)</td>
<td></td>
<td>0.71 (2.13)</td>
</tr>
</tbody>
</table>

**Table II**: Antibiotic resistance pattern and multiple antibiotic resistance index of 29 generic enterococcal isolates from horses

CHL: Chloramphenicol; RIF: Rifampicin; ERY: Erythromycin; VAN: Vancomycin; TET: Tetracycline; MARI: Multiple antibiotic resistance index
(resistance to 2 or more antibiotics) patterns were exhibited by the isolates with ERY-RIF and ERY-TET-RIF (n = 5 for each) being the predominant. MARI of 0.14, 0.29, 0.43, 0.57 and 0.71 was observed for 4, 7, 8, 7 and 3 of the isolates, respectively. Mean MARI for isolates was 0.40 (range 0.14-0.71). Twenty five (86.2%) of the isolates had MARI > 0.2

**OCURRENCE OF GENERIC VANCOMYCIN-RESISTANT ENTEROCOCCI IN HORSES**

Out of 30 isolates, all the 7 that exhibited resistance to high-level VAN disc test were also VAN-resistant in agar screening while those that were intermediate-susceptible in disc test exhibited susceptibility to VAN in agar screening. Of the 7 VAN-resistant isolates, 3 (42.9%) were haemolytic strains while 4 (57.1%) were non-haemolytic.

**Discussion**

The isolation of enterococci from 97% of sampled horses suggested that Slanetz and Bartley agar could be a highly sensitive medium for selective isolation of enterococci from rectal swabs obtained from horses [20]. It also indicated that these organisms colonize the intestinal tract of almost all the horses in Nigeria. This finding is not surprising because enterococci constitute part of normal commensal flora of horses [19]. However, the isolates may also be pathogens and of exogenous origin in the sampled horses [29]. Isolation of 6.2% haemolytic enterococcal strains against 93.8% non-haemolytic strains in this study, suggested that the latter species may be the dominant strains associated with horses in Nigeria. This finding may be attributed to the health status (apparently-healthy) of the horses [3]. The haemolytic and non-haemolytic species in this study may belong to any of the 5 branches/groups (except E. canis) of enterococcal species [36, 43], most of which have been documented to colonize horses [19, 28, 35]. The 97% enterococcal occurrence in this study is higher than 78.9 and 17.4% enterococcal occurrence in faecal samples of 90 purposively-selected mature Lusitano horses in an equestrian centre in Portugal [28] and 637 faecal samples of randomly-selected horses in horse-riding centers in Korea [19], respectively. But it is lower than 100% enterococcal occurrence in 16 faecal samples of randomly-selected horses reported by Thal et al. (1995) [35] in America. It is worth to note that in Nsukka Enugu State, Southeast Nigeria, Anyanwu and Obetta (2015) [3] reported 98.89% enterococcal occurrence in rectal swabs of 95 systematic randomly-selected cattle while in Zaria Kaduna State, northcentral Nigeria, Ngbede et al. (2017a) [30] reported 48.5% and 30.8% enterococcal occurrence in cloacal and rectal swabs of 130 each of randomly-selected poultry birds and cattle, respectively. The variation in the results of these studies may be related to the method of sampling, isolation/processing, medium used for isolation, rate of infection of sampled animals and management of different animal species.

Detection of 7 (26.7%) VAN-resistant isolates among 30 non-repetitive isolates indicated that these horses are potential reservoirs of VRE. It also suggested that either the horses already harboured the VRE or that the isolates acquired the genes encoding VAN resistance from other enterococci in the gut. The use of antibiotics, including VAN, in horses and other animals are not regulated in Nigeria [7]. Thus the findings also suggested that these horses might have been treated with VAN during previous visitations to hospital or by their owners/handlers as well as non-professionals without veterinary supervision. It is also possible that they have been treated with VAN or have acquired VRE while awaiting slaughter at the study site. Unfortunately, there is no pre-slaughter assessment to determine the possible use of VAN in these horses in Nigeria [4].

This finding of VRE calls for concern because these horses potentially serve as reservoirs and disseminators (by faecal shedding) of these organisms into the environment, thereby posing health threat to individuals who get in contact with them [4]. Following contact with faeces from the horses or formites contaminated by the organisms, individuals (such as veterinarians, jockeys, owners/handlers) who often make contact with these horses, could acquire these organisms which easily transfer VAN resistance genes to other bacteria by horizontal transfer [36]. Since the horses are for slaughter and there are poor hygienic practices employed during animal slaughter in Nigeria, consumers, butchers and slaughterhouse workers (especially those who ingest raw horse meat) could acquire these organisms following contact with and/or consumption of meat and associated products from these horses [12, 15, 41]. These individuals could serve as disseminators of these organisms to the public [4]. VAN is a critically-important antibiotic used in management of severe infections in humans and animals; therefore, acquisition of VRE could jeopardize therapy resulting in increased health cost, morbidity and mortality [1]. In India, Singh (2009) [33] used high-level VAN (HLV) disc and observed 80.2% VAN resistance among 267 enterococcal isolates from healthy/sick horses, a finding that is higher than the result (26.7%) of this study. In Belgium, Devriese et al. (1999) [14] detected 8 VRE enterococci in 83 non-duplicate samples of horses using VAN-amended medium and molecular method while in Italy, Niederhausen et al. (2007) [13] observed 7 VRE in 104 samples of healthy horses also using similar methods. Thal et al. (1995) [35] recorded 1VAN-resistant isolate among 16 faecal enterococcal isolates from horses in America using 8 µg/mL of VAN. These results are lower than the findings (7 VRE in 30 non-duplicate samples/non-repetitive isolates) of this study. Differences in these studies could be related to variation in the number and type of samples analyzed, method of VAN resistance detection (whether phenotypic or molecular), concentration of VAN in the test/detection medium, health status of sampled horses, degree of contamination of horses’/animal’s environment and colonization, and degree of usage of avoparcin/VAN in the study area. In the hereby presented experiment, VAN resistance was assessed using HLV (30 µg) disc and
agamizymes level disc test. The authors equally detected pooled enterococcal isolates from poultry/cattle using high-level aminoglycosides resistance in horses should be maintained, because the eventual emergence of HLAR resistance in this study whereas Kim et al. (2016) [19] reported 25% (4/16) HLV resistance, but this glycopeptide has never been used in Nigeria [30]. Therefore, the selection against VAN in this study, could also have resulted due to VAN use in humans and/or other animal species since the drug is readily available over-the-counter in Nigeria. It is worth to note that in Ogun State Southwest Nigeria, Ayeni et al. (2017) [6] reported 65% VAN resistance among 60 enterococcal isolates from healthy poultry using HLV disc method while Ngbede et al. (2017a) [30] did not observe VAN resistance among 167 enterococcal isolates from poultry and cattle in Zaria northcentral Nigeria using the same method.

The absence of HSL and HLG resistance in this study suggested that aminoglycosides have not been abused in equine practice in Nigeria. It equally suggested that the isolates might not have been exposed to sources for acquisition of genes encoding resistance to these agents. Thal et al. (1995) [35] reported 25% (4/16) HLS (> 2,000 µg/mL) and HLG (≥ 256 µg/mL) resistance while Kim et al. (2016) [19] reported 1.6 and 0.5% HLS and HLG resistance, respectively, using high aminoglycosides level disc test method; these results contrasted the findings (0.0% for both drugs) of this present study. The differences in the results of these studies could be related to variation in the use of aminoglycosides in management of horses/animals in the study areas and method of analysis. In this study, the recommended high level aminoglycoside agar screening method was used [10, 11]. It is possible that increasing the number of isolates analyzed in this study might also have increased the likelihood of detecting high-level aminoglycosides-resistant (HLAR) strains.

However, considering the critical relevance of aminoglycosides (in combination with β-lactam) in the management of enterococcal infections, the status of absence of high-level aminoglycosides resistance in horses should be maintained, because the eventual emergence of HLAR enterococci in equine setting in Nigeria, could result in fast spread of these organisms in animal and human population in the country [1, 18]. This will consequently limit therapeutic options available for treatment of enterococcal infections in Nigeria [21]. It is also worth to note that Ngbede et al. (2017a) [30] reported 32.7% HLG and 28.3% HLS resistances among pooled enterococcal isolates from poultry/cattle using high-aminoglycosides level disc test. The authors equally detected the genes codifying the resistances in these isolates [31]. These findings may suggest that poultry and cattle in Nigeria are colonized with HLAR enterococci more than horses.

The moderate to high rates of resistance observed against CHL (36.7%), ERY (80%), TET (50%), and RIF (90%) in this study suggested selection pressure. These resistances may be due to acquisition of genes encoding resistance to the agents following use-selection pressure. Enterococci are known to rapidly acquire and transfer (by horizontal means) genes encoding resistance to almost all available antibiotics [1, 23]. Because sampled horses in this study were used in the northern region and transported to the study site, it was not possible to trace their medical history. Nonetheless, it is possible that they might have been treated earlier with these drugs. It is equally possible that the horses might also have been treated with the drugs at the study site [4]. The 50% TET resistance in this study is higher than 9.8, 40.8 and 18.9% TET resistance recorded among 90, 267 and 190 enterococcal isolates from horses in Portugal [28], India [33] and Korea [19], respectively. The authors equally observed 12.7, 65.9 and 2.6% ERY resistance, respectively; these results are lesser than the findings (80%) of this study. The 36.7% CHL resistance in this study is higher than 0.5% CHL resistance reported by Kim et al. (2016) [19], but it is lesser than 59.2% CHL resistance observed by Singh (2009) [16]. Variation in resistance observed in these studies may be attributed to the use of some of these antibiotics in management of horses and other animal species and may reflect their use in the country [41]. The high CHL resistance in this study calls for serious concern because this antibiotic has been banned for use in animals dedicated for meat in Europe and many other countries [9].

The 62.1% multidrug resistance observed in this study suggested that a high number of enterococci colonizing horses in Nigeria are MDR. This finding is worrisome because of the impact of MDRE on antibiotic therapy and epidemiology of VAN and antibiotic resistance [36]. This high multidrug resistance is a cause for concern because these horses are potential disseminators of MDRE in the environment. These organisms are reservoirs of genes encoding resistance to many classes of antibiotics (including glycopeptides) and they persist in the environment, thereby posing threat (compromise therapy in colonized/infected individuals) to public health [1]. Singh (2009) [33] reported 99.6% multidrug resistance which is higher than that (62.1%) of this study whereas Kim et al. (2016) [19] reported 2.6% multidrug resistance which is lesser than the result of this study. A MARI > 0.2 indicates a ‘high-risk’ source of contamination [22], the mean MARI in this study was 0.41.

Conclusions

This study has shown that MDR- (62.1%) and VRE (23.3%) are harboured by a sizeable percentage of horses slaughtered at Obollo-Afor market in Udenu L. G. A.,
Southeastern Nigeria. Non-haemolytic species are the dominant strains colonizing horses in the study area. Thus these horses are potential reservoirs and disseminators of MDR-/VRE and genes encoding resistance to many classes of antibiotic and VAN. This has huge impact on the food chain, ecology and epidemiology of antibiotic resistance. Therefore attention should be paid on the use of antibiotics, including VAN, in horses in Nigeria. However, further studies to determine the genes encoding VAN resistance in the isolates are recommended.

References


