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Virulence Genes in Gram-negative Enteric Pathogens Isolated from Surface Water Sources in Adamawa North Senatorial Zone, Nigeria

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ABSTRACT

Groundwater sources such as rivers and wells remain the major water supply sources for homes in developing countries' rural areas. They are subject to anthropogenic activities such as domestic, agricultural, and industrial waste disposal. This could pose huge health risks. Rivers and wells in Adamawa North Senatorial Zone of Nigeria were screened bacteriologically to assess their quality. Samples were screened by the membrane filtration method for enteric pathogens and isolates identified by standard bacteriological and molecular methods after which they were screened for the presence of some essential virulence determinants. *Escherichia coli* was the most commonly isolated being recovered from 34.4% and 14.8% respectively of the river and well water samples. Others were *Vibrio cholerae, Vibrio parahemolyticus, Salmonella enterica,* and *Shigella dysenteriae. Shigella dysenteriae*, *Vibrio cholerae*, and *Vibrio parahaemolyticus* were not recovered from the well water samples. Virulence genes detected in *E. coli* were *stx*1, 13(48.2%), *stx*2, 22(81.5%), *eae*A, 21(77.8%), and *hly*A, 15(55.6%). The gene for cholera toxin, *ctx*AB was detected in only 2(28.6%) of the *V. cholerae* isolates. In comparison, hemolysin gene *hly*A was detected in both *V. cholerae* (14.3%), *V. parahaemolyticus* (16.7%), *S. enterica* (40.0%), and the only isolate of *S. dysenteriae*. Detecting enteric pathogens with some putative virulence genes indicated a health risk to consumers. Intensive health education, good sanitary habits, and point-of-use water disinfection are advocated for consumers.

INTRODUCTION

Access to safe drinking water is of utmost importance to human health as this is crucial to the prevention of water-borne diseases. Globally, fresh water is becoming a limited resource due to population expansion, contamination from anthropogenic sources and climate changes (Hassan Rashid *et al.*, 2018). The supply of clean drinking water is reported to be one of the major challenges facing most African countries (Naik, 2017). In most developing countries, most residents either drill boreholes, buy water from vendors in tanks, dig shallow wells, or depend on rainwater or water from rivers (Yusuf, 2007; United Nations, 2014).

The issue with these sources is that they are not treated and most often do not meet the World Health Organization (WHO) water standards (United Nations, 2014). Reports show that water-related diarrhoea illness alone is among diseases that cause high morbidity and mortality, killing 1.8 million people and causing approximately 4 billion cases of illness annually (Azuonwu *et al.*, 2017).

Developing countries are reported to be among the worst afflicted with water-borne disease outbreaks (WHO, 2018) some of which, such as typhoid fever are endemic (Ajibola *et al.*, 2018). Most rural dwellers consume water without testing, bothering about its portability as they believe that dirt cannot harm them. Recent studies however disapprove of this fact as diseases such as cholera, diphtheria typhoid fever have been linked to the consumption of contaminated water (Sur *et al.*, 2006). Cholera outbreaks have been reported in Zimbabwe, India and Nigeria which were caused by the presence of *V. cholerae* in municipal taps and wells (Sur *et al.*, 2006). Drinking water from river Zamani was implicated in the cholera outbreak of 2014 in Gomani, Kwali Local Government Area in the Federal Capital Territory of Nigeria (Dan-Nwafor *et al.*, 2019). Hand-dug well water has also been implicated in typhoid outbreaks in Pakistan (Farooqui *et al.*, 2009). Several reports have actually documented the presence of pathogenic and potentially pathogenic bacteria such as *E. coli*, *Salmonella enterica*, *Shigella* spp, *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Pseudomonas aeruginosa* in water sources in some developing countries (Raji *et al.*, 2010; Ayandele *et al.*, 2019; Enabulele *et al.*, 2022). A few have also reported the presence of virulence genes *stx1*, *stx2* and *VacA* from freshwater biofilms (Soborg *et al.*, 2013).

A comprehensive evaluation of the microbial quality of water requires a survey of all pathogens that have the potential for human infections (Miranzadeh *et al.*, 2011). Data on the quality of water sources in Adamawa North Senatorial Zone is scarce and this study is aimed at augmenting this scarce data.

MATERIALS AND METHODS

Study Area:

This investigation was carried out at Adamawa North Senatorial Zone which consists of five Local Government Areas, namely: Madagali, Michika, Mubi South, Mubi North and Maiha (Fig. 1). It has a land size of 4,493.815 km^2 and a population of 682,026 (NPC, 2010). It is located between 9^o 30’ and 11^o 00’N of the equator and between 13^{o}00’ and 14^{o}00’ of the Greenwich Meridian. The dry season lasts for a minimum of 6 months spanning between November and March while the wet season spans between April and October (Tula et al., 2022).

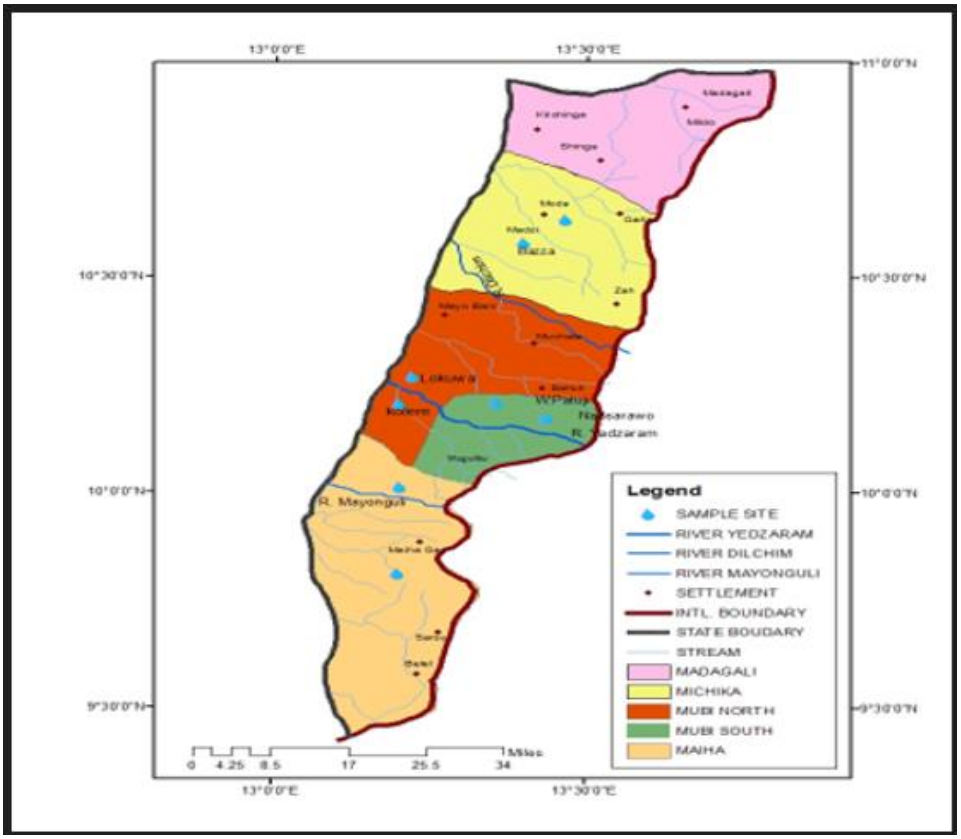

Fig.1: Map of Adamawa-north senatorial zone

Collection of Samples:

Raw water samples were collected in clean sterilized 50ml glass bottles from River Yedzaram, Dilchim, and Mayonguli as well as 32 hands dug wells all in the Mubi zone of Adamawa State, Nigeria with the aid of strings and metal weights tied to the bottles. Samples were collected at about 1m depth below the water surface. Four (4) wells were sampled in each of the eight (8) wards that constitute the zone and samples were taken during the dry and wet seasons making a total of 128 samples. Sixteen samples were collected from Rivers Dilchim and Mayonguli, eight each during the dry season and wet seasons. Thirty-two (32) were screened in River Yedzaram as this River runs through two local government areas of Mubi North and South, 16 samples respectively from each Local Government Area. Samples were kept in ice-cold boxes and taken to the laboratory for analysis.

Bacteriological Analyses:

Enteropathogens were isolated by culturing samples on plates of MacConkey agar, Salmonella Shigella agar and Thiosulphate citrate bile salts agar, using the membrane filtration technique. The cultures were incubated at 37 ^{o}C for 18 – 24h after which isolates were purified on fresh agar plates and stored in nutrient agar slants for further analysis. The purified isolates were subjected to Gram staining and then identified by both cultural and conventional biochemical tests (Cheesbrough, 2006) and Microgen Gram-negative A (GN-A) kits.

Detection of Virulence Genes:

Genes that play relevant roles in the pathogenicity of the species identified were screened for using the polymerase chain reaction (PCR) with specific primers. Genes for Shiga-like toxins 1 (*stx1*), 2 (*stx*2), attaching and effacing gene (*eaeA*) and hemolysin A (*hlyA*) in *E. coli* were investigated using the primers as shown in Table 1.

Shigella dysenteriae was also screened for *stx*1 and *stx*2 and *hly*A as well as invasive genes (*Inv*A). *Salmonella enterica* isolates were screened for invasive genes *Inv*A *and*

*hly*A while the *Vibrio* spp were screened for *hly*A and cholera toxin (*ctx*AB) genes (Table 1).

The PCR profiles were done by preliminary denaturation for 5mins at 94^{o}C and then 30 rounds of denaturation for 30 seconds at 94^{o}C, hardening for 60 seconds at 50^{o}C and lengthening for 30 seconds at 72^{o}C. The products were taken through 2% agarose gel electrophoresis, with the gel stained with ethidium bromide and examined under an ultraviolet transilluminator.

Table 1: Targeted virulence genes

Target genes	Primers	Ref
Shiga toxin 1 gene (*stx*1)	F:(ACACTGGATGATCTCAGTGG) R:(CTGAATCCCCCTCCATTATG) Targeting 614bp	(Tahamta and Namavari, (2014)
Shiga toxin 2 gene (*stx*2)	F:(GGCACTGTCTGAAACTGCTCC) R:(TCGCCAGTTATCTGACATTCTG) targeting 255bp	Paton and Paton, 1998
Intimin gene (*eae*A)	F: (GACCCGGCACAAGCATAAGC) R: (CCACCTGCAGCAACAAGAGG) targeting 384bp	Paton and Paton, 1998
Hemolysin A (*hyl*A) gene	F:(GCATCATCAAGCGTACGTTCC) R:(AATGAGCCAAGCTGGTTAAGCT) targeting 534bp	Paton and Paton, 1998.
Invasive E gene (*InvE*)	F:(GGATCCATGATTCCTGGTTCCACC TCC) R:(AAGGTTTTAAGACGGCTTTTCAAT AGTACGA) targeting 1119bp	Singh *et al*. 2018
Invasive A gene (*Inv*A)	F:(GTGAAATTATCGCCACGTTCG GGCAA) R: (TCATCGCACCGTCAAAGGAACC) targeting 280bp	Paton and Paton, 1998.
Enterotoxin (*ctxAB*) gene	F: (GCCGGGTTGTGGGAATGCTCCAAG) R:(GCCATACTAATTGCGGCAATCGCATG) targeting 534bp	Goel, 2007

RESULTS

The enteric pathogens isolated from the two water sources are shown in Table 2. Eighty-two (82) out of the 256 samples harboured potentially pathogenic bacteria. *Escherichia coli* was the most prevalent, being recovered from 44 (34.4%) and 19 (14.8%) samples from rivers and wells respectively. *Shigella dysenteriae* was the least isolated as it was found in only one river water sample. *Salmonella enterica* was isolated from a total of 10 samples, while *V. cholerae* and *V. parahaemolyticus* were recovered from 7 and 6 samples respectively and only from river water samples. The Shiga-toxin (*stx*1 and *stx*2) genes were detected in 13 (48.2%) and 22 (81.5%) of *E. coli* isolates. Eleven isolates of *E. coli* had both *stx*1 and *stx*2 genes. They also had *eae*A (77.8%) and *hly*A (55.6%) genes. The intimin (*eaeA*) gene was also detected in two isolates without the other virulence genes. The only isolate of *S. dysenteriae* had only *hly*A gene and no Shiga-like toxin with *stx* genes was detected. Two (28.6%) of the *V. cholerae* isolates had genes for cholera toxin, and *ctx*AB and one of the isolates also had the *hly*A gene. The *Inv*A gene was detected in 4(80.0%) of the *S. enterica* isolates, two (40.0%) of which also had the *hly*A gene (Table 3).

Table 2: Prevalence of Gram-negative enteric pathogens in water sources in Adamawa-north Senatorial Zone, Adamawa State

Bacterial spp	**Number (%) of positive samples**		**Total (n = 256)**
	Rivers (n = 128)	Wells (n = 128)	
Escherichia coli	44 (34.4)	19 (14.8%)	63 (24.6)
Vibrio cholerae	7 (5.5)	0 (0.0)	7 (5.5)
Vibrio parahaemolyticus	6 (4.7)	0 (0.0)	6 (23)
Salmonella enterica	4 (3.1)	1 (0.8)	5 (20)
Shigella dysenteriae	1 (0.8)	0 (0.0)	1 (0.4)
Total	62 (48.4)	20 (15.6)	82 (32.0)

Table 3: Virulence genes in Gram-negative enteric pathogens from water sources in Adamawa-north Senatorial Zone, Adamawa State

Putative Virulence gene	**Number (%) of positive isolates**				
	E. coli (n = 27)	*V. cholera* (n = 7)	*V. parahaemolyticus* (n = 6)	*S. enterica* (n = 5)	*S. dysenteriae* (n = 1)
*stx*1	13 (48.2)	NA	NA	NA	0 (0.0)
*stx*2	22 (81.5)	NA	NA	NA	0 (0.0)
*eae*A	21 (77.8)	NA	NA	NA	0 (0.0)
*hly*A	15 (55.6)	1 (14.3)	1 (16.7)	2 (40.0)	1 (100.0)
*Inv*A	NA	NA	NA	4 (80.0)	NA
*ctx*AB	NA	2 (28.6)	0 (0.0)	NA	NA

KEY: N = number of isolates, NA = Not Applicable, *stx* = Shiga-like toxin, *eae*A = *E. coli* attaching and effacing (intimin) gene, *hly*A = Alpha haemolysin gene, *Inv*A= Invasive A gene, *ctx*AB = Cholera toxin gene.

DISCUSSION

The results of this study highlighted the presence of bacteria with some putative virulence genes relevant to gastrointestinal tract infection in the water sources screened. The presence of enteric pathogens such as *E. coli*, *Salmonella enterica*, *Vibrio cholerae* and *Vibrio parahaemolyticus* in water sources in Nigeria and other developing countries is well documented in the literature (Enabulele and Aikpitanyi-Iduitua, 2003; Raji *et al.*, 2010; Olalemi *et al.*, 2021) and is similar to the isolates obtained in this report. Their detection may suggest a continual spread of enteric bacteria with the potential of causing serious enteric infections, particularly among children, the elderly and the immunocompromised. *stx*1, *stx*2, *eae*A and *hly*A amplicons were all detected in some of the isolates of *E. coli*. *stx*1 and *stx*2 genes mediate the synthesis of Shiga toxins which are virulence determinants for shiga-toxin producing/ Enterohaemorrhagic *E. coli* (STEC/ EHEC) and *Shigella dysenteriae*. These are pathotypes of *E. coli* that cause severe disease in humans including bloody diarrhea and haemolytic uraemic syndrome (Orth and Wurzner, 2006). Shiga-like toxins are reported to destroy microvascular endothelial lining leading to hemorrhagic clots and haemolytic uraemic syndrome, with the input of other virulence genes (Mellmann *et al.*, 2006). Such additional genes include *eae*A gene which encodes intimin that helps in attachment to host cells and the haemolysin gene *hly*A (Karch *et al.*, 2006) amongst others. The results here are also similar to previous reports that have documented the presence of *stx*1, *stx*2 and *eae*A amplicons in *E. coli* recovered from drinking water sources such as wells (Odetoyin *et al.*, 2022), rivers and

streams (Sidhu *et al*., 2013; Titilawo *et al*., 2015; Enabulele *et al*., 2022). The detection of both *stx*1, *stx*2 amplicons in some *E. coli* isolates is a cause for serious concern as strains carrying this combination have been reported to cause more complicated diarrhea in men (Paton and Paton, 1998; Titilawo *et al.*, 2015). The two isolates of *E. coli* possessing only the *eae*A gene but lacking the other genes which are typical of the STEC pathotype probably belong to the enteropathogenic *E. coli* (EPEC) group. This finding is similar to a previous report in South Western Nigeria where a few of the *E. coli* isolates from a river harboured only the *eae*A gene but lacked other typical virulence genes of the EPEC group. The presence of the attaching and effacing (A/E) lesion mediated by the *eae*A protein intimin, and the absence of Shiga toxins is distinctive of the EPEC group. Typical EPEC bear *eae*A and *bfp*A (bundle forming pili gene) (Kaper, 1996) while *eae*A alone is a characteristic of the atypical EPEC. One limitation of this study is the inability to differentiate the isolates into typical and atypical EPEC. However, the detection of *E. coli* strains with only the *eae*A gene is also a health risk as such strains have been reported to cause diarrhea in some studies, especially in children (Snehaa *et al.*, 2021). Studies showed that EHEC strains with *eae*A gene are more virulent compared with those that lack it (Shojaei, 2017). The *hly*A gene was detected in some of the *E. coli* strains (Miranzadeh *et al.*, 2011), one each of the *V. cholera* and *V. parahaemolyticus*, the only *S. dysenteriae* isolate and two *S. enterica* isolates. The *hly*A gene codes for enterohaemolysin, a pore-forming toxin that causes lysis of red blood cells and nucleated host cells when present in high concentration (Russo *et al.*, 2005).

The concentration of EHEC hemolysin which is encoded by the *hly*CABD operon, in which *hly*A is the structural gene, in the pathogenesis of EHEC is increasingly being recognized. EHEC *hly*A exists in the two biologically active forms. While the free-form lyses human microvascular endothelial cells, the one bound to the outer membrane targets the mitochondria causing apoptosis (Bielazewska *et al.*, 2014). The *stx* gene was absent in the only *S. dysenteriae* isolate. This isolate may be any, other than type 1 *S. dysenteriae* which is known to harbor *stx* genes. Type 2 and other strains may not bear this gene, as an earlier report showed that all 113 strains *S. dysenteriae* Type 2 isolated from diarrhoea patients in Dhaka, Bangladesh did not have *stx* genes (Talukder *et al.*, 2006). The presence of *Inv*A gene in all but only one of the *S. enterica* isolates tallies with a report that *Inv*A was the most common virulence gene detected in *Salmonella* (Thung *et al.*, 2018). *Inv*A gene mediates the production of a protein in the bacterial cell membrane that helps bacteria invade and survive in host cells.

Cholera toxin genes *ctx*AB genes were detected in only the *Vibrio cholerae* isolates. This low prevalence of *ctx*AB gene is consistent with earlier reports (Alam *et al.*, 2006; Akoachere *et al.*, 2013) of the low prevalence of toxigenic *V. cholerae* in environmental samples. However, these isolates could also pose health threats as several reports have implicated non-toxigenic strains of *V. cholera* in mild to severe cholera-like diarrhoea in men (Dutta *et al.*, 2013; Kumar *et al.*, 2018). The *hly*A gene was detected in one isolate each of *V. cholerae* and *V. parahaemolyticus.* It mediates a pore-forming toxin that causes cell lysis and also lengthens the stay and pathogenesis of *V. cholerae* in epithelial cells (Fu *et al.*, 2020).

Conclusion:

This study highlighted the presence of enteric pathogens in well and river water samples with putative virulence genes that can pose serious public health threats to consumers. It is recommended that water wells should be cited in locations with no easy access to sources of contamination and these facilities should be well maintained and protected from domestic animals and runoffs. Users of surface waters and wells

should be sensitized to the need for point-of-use disinfection such as boiling for a few minutes, especially that meant for drinking purposes.

Declarations:

Ethical Approval: Not necessary

Conflicts of Interest: The authors declare no competing interests.

Authors Contributions: All authors were responsible for the study design, experiment execution, data analysis, and manuscript drafting.

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Availability of Data and Materials: All datasets analyzed and described during the present study are available from the corresponding author upon reasonable request.

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