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Full Length Research Paper

Efficacy of two diagnostic tests for the detection of infectious bursal disease viruses in chicken from different types of bursal samples

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Infectious Bursal Disease (IBD) is an economically important irreversible immunosuppressive disease of young birds. The present study was designed to confirm the efficacy of two common diagnostic tests for the detection of Infectious Bursal Disease Virus (IBDV) from the three types of bursal samples collected from a recent outbreak in layer and broiler chickens of Gazipur district, Bangladesh. This study compared the degree of sensitivity between Ouchterlony Double Immunodiffusion Test (ODIT) and Reverse Transcription Polymerase Chain Reaction (RT-PCR) for the detection of IBD viral antigen from the bursal samples. A total of 180 field bursal samples (80 broiler and 100 from layer chicken) from suspected IBDV infected dead chickens were collected from 50 different poultry farms. Bursal homogenates were used to detect IBDV using ODIT and RT-PCR. Three types of bursal samples, hemorrhagic bursa (90), edematous bursa (78) and atrophied bursa (12) were selected for the detection of viral antigen. A panel of anti-sera and IBDV specific primer for VP2 gene was used for RT-PCR. The data demonstrated that, out of 180 field samples, 164 (91%) were positive by RT-PCR and 120 (67%) were positive by ODIT. Haemorrhagic bursas were more sensitive compared to oedematous bursas while no virus was detected from the samples of atrophied bursa. This study demonstrated that, RT-PCR was more sensitive and effective diagnostic tool compared to that of ODIT.

Key words: Infectious bursal disease virus (IBDV), ouchterlony double immunodiffusion test (ODIT), reverse transcription polymerase chain reaction (RT-PCR), bursa, broiler, layer.

INTRODUCTION

Infectious bursal disease or Gumboro is a vastly transmissible immunosuppressant disease which is triggered with a virus belonging to the genus *Avibirnavirus* of family Birnaviridae. IBD is associated with high

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mortality affecting mainly 3 to 6 weeks old age group but subclinical and less acute form is also mutual in 0 to 3 week old chickens (Teshome and Admassu, 2015). IBD can cause an acute viral disease with 80 to 100% morbidity and mortality that varies from 20 to 30% in broilers whereas it is 40 to 80% in layers, respectively, depending on the strain virulence (Van den Berg et al., 2000; Chowdhury et al., 1996). There are three different pathotypes of IBD virus namely, very virulent (vvIBDV), classical virulent (cIBDV) and variant (Michel and Jackwood, 2017), the later one being responsible for prolonged immunosuppression. The clinical form of IBD is associated with significant economic losses with irreversible immune suppression, reduced effectiveness of vaccination, retarded growth, poor feed consumption, decreased egg quality and decreased egg production. In addition, the excessive hemorrhages within the muscles consequences in increased mortality of the chickens. The characteristic clinical sign of the IBD infected chicken includes whitish watery droppings followed by depression, anorexia, severe prostration, trembling and finally death (Sali, 2019). Virus strain, age and also the breeds of chickens affect the severity of the disease (Teshome and Admassu, 2015). Infection with less virulent strains may not show remarkable clinical signs besides the chickens may have fibrotic or cystic bursa of Fabricius that might turn into atrophied prematurely (before six months of age). During postmortem diagnosis, the chickens showed hemorrhages in the pectoral or thigh muscles, dehydration, urate deposits within the kidneys, enlarged, edematous, hyperemic and or atrophic bursa of Fabricius (Aliyu et al., 2016; Khan et al., 2009). In chronic cases, presence of hemorrhage within the connection between gizzard and proventriculus is observed (Khan et al., 2017; Aliyu et al., 2016). The virus generally infected the lymphoid tissue especially the bursa (Dey et al., 2019; Wang et al., 2011; Van den Berg et al., 2000) and thus others immune organs like as spleen, bone marrow, thymus are also involved. Several diagnostic methods are used for the identification of IBDV through various levels of specificity and sensitivity (Daodu et al., 2018; Oluwayelu et al., 2014; Okwor et al., 2011; Hussain et al., 2003; Nachimuthu et al., 1995; Aliev et al., 1990; Allan et al., 1984). Common serological tests such as agar gel immunodiffusion test (AGIDT), enzyme-linked immunosorbent assay (ELISA), immunofluorescent test and indirect hemagglutination (IHA) test are being used for the detection of IBDV (Daodu et al., 2018). Although, the confirmatory diagnosis of IBDV is very important for formulating an effective strategy intended for control of the disease. ODIT is also familiar as passive double immunodiffusion or AGID test. ODIT is a simple method to detect specific antibody and measure antigenicity (Hornbeck, 2017). It is simplest but least sensitive serological method (Sali, 2019). It measures primarily group specific soluble antigens and cannot distinguish serotypic variances. It can be used to produce a precipitin line by estimating antibody size through dilutions of serum in the test well and taking the titer as the highest dilution (Sali, 2019). The molecular methods like RT-PCR is used to amplify the reverse transcription of the DNA code (Saiki et al., 1985). RT-PCR is the method which helps for precise and early diagnosis of viruses from field samples (Hasan et al., 2010). Among these diagnostic methods, in this study we chose ODIT and RT-PCR as ODIT is simplest of all, inexpensive and has the ability for detecting specific antibodies (Dey et al., 2019; Hornbeck, 2017; Ouchterlony, 1948; Ouchterlony and Nilsson, 1986) and RT-PCR is specific, sensitive, speedy, reliable and accurate techniques for detection of IBD viruses (Hasan et al., 2010).

IBD is an economically significant disease in poultry sector. The irreversible immune suppression caused by IBD virus in young chicks increases their susceptibility to an assembly of opportunistic avian pathogens that are normally non-pathogenic in healthy groups (Michel and Jackwood, 2017). Usually the farmers are worried about the present monetary mortality value from the lost flock and never see beyond if the chickens were to get relief from the disease. These collectively consequences remarkable economic losses for the poultry farmers which are often high and alarming if not properly diagnosed. Fresh samples from the affected chickens are usually more suitable to detect the virus but in rural areas of Bangladesh, there is very limited opportunity for the identification of poultry disease at the field level. The diagnosis is done mainly based on the clinical history, sign-symptoms and necropsy findings where there is a high possibility of misinterpretation and wrong diagnosis, because many poultry diseases produce similar clinical signs, symptoms and postmortem findings which are sometimes very difficult to differentiate (Hasan et al., 2010; Sali, 2019). Therefore, this study was designed to compare the sensitivity of the molecular technique (RT-PCR) and serological technique (ODIT) for the detection of IBDV from three types of bursal samples.

MATERIALS AND METHODS

Ethical approval

The research was carried out following the guidelines set forth by the Animal Welfare and Experimentation Ethics Committee of Bangladesh Agricultural University, Mymensingh, Bangladesh (ref. no. AWEEC/BAU/2019(38)).

Sample collection

A total of 180 (broiler 80 and layer 100) bursa of Fabricius (BF) (hemorrhagic, edematous and atrophied bursa) were collected from a recent outbreak in broiler and layer chickens of Sadar Upazila in Gazipur district, Bangladesh.

Sample preparation

Three types of bursal samples were macerated separately with

sterilized mortar and pastel to prepare 10 to 20% (w/v) suspension in sterile PBS. The suspension was centrifuged at 4000 rpm for 30 min to separate tissue debris from the supernatant. The supernatant thus obtained was treated by a broad-spectrum antibiotic (Gentamycin) at 50 µg/ml and the prepared inoculates were tested for bacterial contamination by culturing 0.1 ml of each on nutrient agar and blood agar plates for 24 h at 37°C.

Hyper immune serum preparation for ODIT

Four non vaccinated chickens were immunized with the vaccine (Nobilis® Gumboro 228E, Intervet International, The Netherlands) orally at 7, 14, 21, 28 and 35 days of age. After 10 days of last vaccination blood were collected to obtain serum which was used as hyper-immune serum in ODIT.

ODIT

Immunodiffusion plates were prepared by melting 8 g of sodium chloride in 100 ml of distilled water followed by the addition of 1.25 g agar noble. The mixture was gently mixed and boiled in a water bath until the agar was absolutely dissolved. The agar was given left to cool at 50°C before it was poured in 6 of 9 cm immunodiffusion plates and allowed to solidify. The plates were then kept overnight at 4°C until used. Applying a template and well cutter (4 mm), seven wells of 4 mm (a group of six wells surrounding a centre well) were made. The central well of the glass slide filled with melted agarose gel was loaded by known hyper-immune sera against IBDV and peripheral wells by bursal suspensions. Slides were kept in moist chamber for 24 to 48 h at 4°C and observed for antigen antibody reaction in the form of appearance of precipitation lines in between the peripheral and central well.

Extraction of viral RNA

Viral RNA of IBD virus was extracted from three types of bursal suspensions using the QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's informations. Then the extracted RNA was treated with RNase-free DNAse I (Fermentas, Canada) and after that using the first strand cDNA synthesis kit, HyperScript (GeneAll), cDNA was synthesized from the treated RNA.

RT-PCR

RNAs from bursal samples were reverse transcribed to cDNA and amplified by a one-step RT-PCR. RT-PCR performed on a MJ Mini Thermocycler, Bio-Rad, USA. Primers Vv-fp775 (forward primer, 5′-AATTCTCATCACAGTACCAAG-3′) and Vv-rp1028 (reverse primer, 5′-GCTGGTTGGAATCACAAT-3′) were used to amplify a 253 bp fragment of the VP2 gene (Hasan et al., 2010). RT-PCR was performed at 42°C for 1 h followed by activation of Taq polymerase at 94°C for 2 min, then 35 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 1 min and elongation at 60°C for 1.5 min. A final extension step at 60°C for 10 min was performed. After amplification, RT-PCR product was subjected to electrophoresis on 2% agarose gel containing ethidium bromide. The band was examined under UV-trans-illuminator.

Statistical analysis

Statistical analyses were accomplished using InStat® software (GraphPad, USA). Correlations between the proposed techniques were analyzed followed by Chi-squared test. Differences were considered statistically significant at *P < 0.05 or ***P < 0.001.

RESULTS AND DISCUSSION

IBDV endures to be a main concern for the commercial and subsistent poultry farmers including an utmost essential threat regarding poultry production systems in Bangladesh. So, in this study, we compared two to identify the effective diagnostic approaches for the detection of IBDV through ODIT and RT-PCR from the three types of bursal samples.

Apparently, no previous reports were found documenting the sensitivity of the tests using three types of bursal samples for virus isolation. ODIT is one of the substitute techniques suggested for IBDV diagnosis by Organization for Animal Health (OIE) for international trade (Butt et al., 2015). The ODIT is the most useful serological technique for the detection of specific antibodies in sera, or for identifying viral antigen or antibodies in bursa. The ODIT is used to detect prominent white line of precipitation between bursal homogenates of the peripheral wells and known positive anti-IBDV hyper-immune serum of the central well due to antigen and antibody reaction (Hornbeck, 2017). Our data demonstrated that, in case of ODIT, hemorrhagic and edematous bursal homogenates were found positive for IBDV. Prominent white line of precipitation was observed between known positive anti-IBDV hyper-immune serum of the central well and bursal homogenates of the peripheral wells due to antigen and antibody reaction within 24 to 48 h which agrees with Abraham-Oyiguh et al. (2015), Okwor et al. (2011) and Roy et al. (2008) findings. By ODIT, out of 180 samples, 120 samples (H.B.S. 34; E.B.S. 17 from broiler and H.B.S. 44; E.B.S. 25 from layer) were positive for IBDV (Table 1 and Figure 1). No line of precipitation was found in the atrophied bursal homogenates that were considered as negative for IBDV antigen. These results suggested that preparation of hyper-immune serum was appropriate and properly functioned to identify the IBDV antigen in ODIT test, while hyper-immune serum is not invariably available and commercially highly expensive. Additionally, hemorrhagic bursa was more effective compared to edematous bursa whereas no virus was detected in atrophied bursa. Previous report suggested that for viral replication, bursa of Fabricious is the vital target organ. In acute case, bursa of Fabricious is haemorrhagic, oedematous, turgid and within 7 to 10 days turns in atrophic (Dey et al., 2019). These differentiations in clinical signs depend on the subsistence of maternal immunity, virulence of causative agent and bird's age (El-Samadony et al., 2019; Rauw et al., 2007; Hassan, 2004). In the bursa of Fabricious, the stage of B cell differentiation keeps a vital role for viral replication as the stem cell (Dey et al., 2019). Our study

Table 1. Rate of identification of IBDV from the three types of bursal samples of broiler and layer chickens.

Sample type	Broiler identification		Sample type	Layer Identification		Comparative sensitivity of identification of IBDV in broiler and layer chickens	
	ODIT (%)	RT-PCR (%)		ODIT (%)	RT-PCR (%)	ODIT	RT-PCR
H.B.S. (40)	34 (85)	40 (100)	H.B.S. (50)	44 (88)	50 (100)		
E.B.S. (34)	17 (50)	32 (94)	E.B.S. (44)	25 (57)	42 (95)	120 (67)	164 (91)
A.B.S. (6)	0 (0)	0 (0)	A.B.S. (6)	0 (0)	0 (0)		

H.B.S. = Haemorrhagic bursal suspension; E.B.S. = Oedematous bursal suspension; A.B.S. = atrophied bursal suspension.

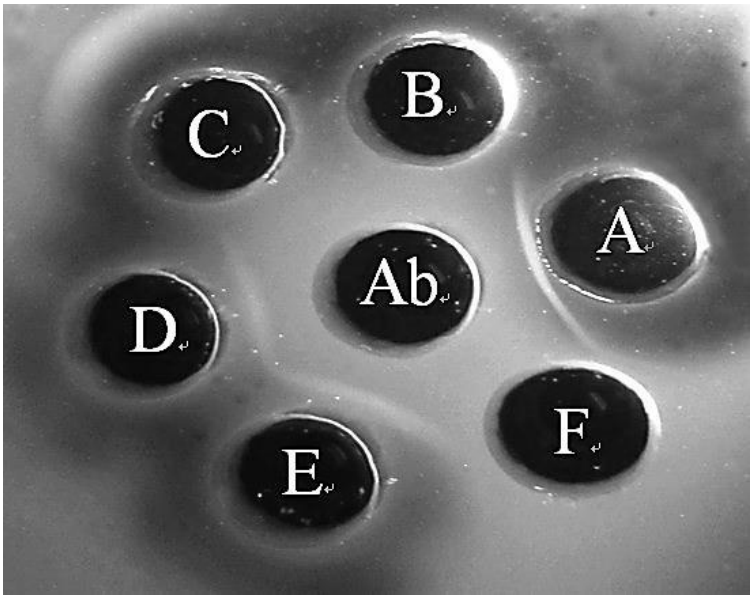

Figure 1. ODIT indicating the occurrence of IBD virus in bursal samples using hyperimmune serum. Ab = Hyperimmune serum against IBDV, A = haemorrhagic bursal suspension, B = atrophied bursal suspension 1, C = atrophied bursal suspension 2, D = oedematous bursal suspension 1, E = oedematous bursal suspension 2, F = mock solution.

observes that, there might be the possible difference among the bursal samples for pathogenesis of IBD were irreversible bursal follicle damage and IgM-bearing B lymphocytes and others cell damage (Rodriguez-Chavez et al., 2002), those were more severe in haemorrhagic bursa compared to oedematous and atrophied bursa which increased the sensitivity in haemorrhagic bursa compared to oedematous and atrophied bursa for IBDV.

RT-PCR is used to detect viral RNA in homogenates of infected organs or embryos without considering the viability of the virus present (Hasan et al., 2010). RT-PCR works on hypervariable region (VP2). The VP2 contains a hypervariable region which displays the greatest amount of amino acid sequence variation between strains. This area is responsible for antigenic variation, tissue-culture adaptation and it is slightly responsible for viral virulence (Escaffre et al., 2013). Finally, in terms of the three types of bursal samples for virus detection through RT-PCR, this study highlighted that out of 180 field samples, 164 samples (H.B.S. 40; E.B.S. 32 from broiler and H.B.S. 50; E.B.S. 42 from layer) were positive for IBDV whereas no virus was detected in atrophied bursa (Table 1 and Figure 2). Moreover, we observed that the association between ODIT and RT-PCR in IBDV affected broiler and layer chickens bursal samples was considered statistically significant (Tables 2 to 4). Amplification of VP2 gene (Zahoor et al., 2010) by RT-PCR is obvious by the presence of 253 bp band (Figure 2). The size and location of the bands for each type of samples were identical. These results were in good coordination with the findings of Zohair et al. (2017), Mawgod et al. (2014), Jackwood and Stoute (2012), Kusk et al. (2005). Generally, RT-PCR plays a vital role in the identification of viral antigens through enzymatic amplification of DNA

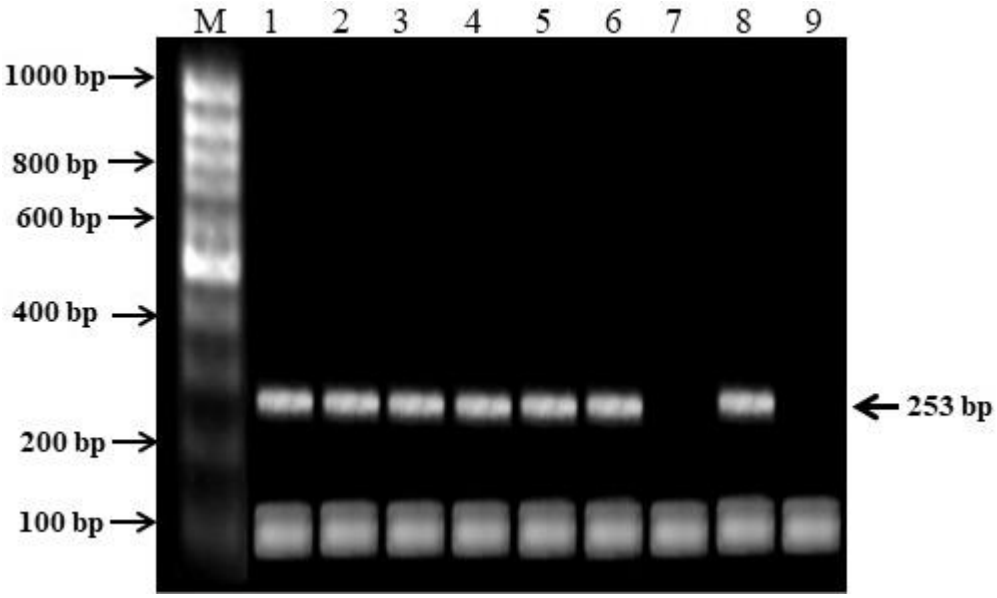

Figure 2. Results of RT-PCR products (253 bp) of IBD virus from bursal samples of chickens analyzed using 2% agarose gel electrophoresis. M = DNA Marker (100 bp), Lane-1 = haemorrhagic bursal suspension 1, Lane-2 = haemorrhagic bursal suspension 2, Lane 3 = haemorrhagic bursal suspension 3, Lane-4 = oedematous bursal suspension 1, Lane-5 = oedematous bursal suspension 2, Lane-6 =oedematous bursal suspension 3, Lane-7 = atrophied bursal suspension, Lane-8 = positive control, and Lane-9 = negative control.

Table 2. Correlation between ODIT and RT-PCR in IBDV affected broiler and layer chickens bursal samples.

Sample types (B + L)	ODIT		RT-PCR		Chi-squared (χ2) value between ODIT and RT-PCR	Degree of freedom (DF)	P-value
	Pos. (B + L)	Neg. (B + L)	Pos. (B + L)	Neg. (B + L)			
H.B.S. (90)	78	12	90	0	10.8035	1	0.0005***
E.B.S. (78)	42	36	74	4	32.3094	1	0.0001***
A.B.S. (12)	0	12	0	12	-	-	-

From each type of bursal samples and for individual diagnostic test, the number of positive samples of broiler and layer chickens were combined together and similarly all number of negative samples of broiler and layer chickens were added together and analyzed using Chi-squared (χ^2) test. ***P < 0.001 indicated the association was considered statistically significant. B = Broiler; L = layer; H.B.S. = haemorrhagic bursal suspension; E.B.S. = oedematous bursal suspension; A.B.S. = atrophied bursal suspension; Pos. = positive; Neg. = negative.

Table 3. Correlation between ODIT and RT-PCR in IBDV affected broiler chickens bursal samples.

Sample type	ODIT		RT-PCR		Chi-squared (χ^2) value between ODIT and RT-PCR	Degree of freedom (DF)	P-value
	Pos.	Neg.	Pos.	Neg.			
H.B.S. (40)	34	6	40	0	4.5045	1	0.0169*
E.B.S. (34)	17	17	32	2	14.3157	1	0.0001***
A.B.S. (6)	0	6	0	6	-	-	-

*P < 0.05 or ***P < 0.001 indicated the association were considered statistically significant. H.B.S. = Haemorrhagic bursal suspension; E.B.S. = oedematous bursal suspension; A.B.S. = atrophied bursal suspension; Pos. = Positive; Neg. = Negative.

code (Kralik and Ricchi, 2017). It is due to identification of important veterinary and clinical viruses through serological approaches are time-consuming or impossible, whereas ODIT is not universally available and sustain from comparatively low specificity and sensitivity. However, this study demonstrated that RT-PCR was more sensitive than ODIT (Mahmood and Siddique, 2006). Previous study reported that RT-PCR give more

Table 4. Correlation between ODIT and RT-PCR in IBDV affected layer chickens bursal samples.

Sample type	ODIT		RT-PCR		Chi-squared (χ^2) value between ODIT and RT-PCR	Degree of freedom (DF)	P-value
	Pos.	Neg.	Pos.	Neg.			
H.B.S. (50)	44	6	50	0	4.4326	1	0.0176*
E.B.S. (44)	25	19	42	2	16.0113	1	0.0001***
A.B.S. (6)	0	6	0	6	-	-	-

*P < 0.05 or ***P < 0.001 indicated the association were considered statistically significant. H.B.S. = Haemorrhagic bursal suspension; E.B.S. = oedematous bursal suspension; A.B.S. = atrophied bursal suspension; Pos. = Positive; Neg. = Negative.

specific and sensitive data than serological methods for amplification of VP2 gene of IBD virus (Van den Berg et al., 2000). RT-PCR again confirmed that, haemorrhagic bursal sample reveals highest effectiveness than the oedematous bursal samples for the detection of IBD antigen. Accordingly, these results suggested that out of the two available methods for IBDV detection, RT-PCR was more efficient than ODIT and the possible reasons might be RT-PCR is highly sensitive diagnostic technique (Vogel et al., 2012) that generates rapid and precise results with amplification of a specific component of DNA (Garibyan and Avashia, 2013).

Conclusions

Confirmatory diagnosis of IBD in both clinical and subclinical cases is very important for the control and prevention of infection in the poultry farms with confirmation of effective strategy as there is a substantial economic loss to the farmers due to IBD. This study will be very helpful for the subsistent poultry farmers in this regard. The study recommended hemorrhagic bursal samples remained highly sensitive for detection of IBD viral antigen compared to that of edematous bursal samples whereas no virus was detected by atrophied bursa. This study suggested that between the two different methods (ODIT and RT-PCR), RT-PCR was highly efficient compared to that of ODIT and hemorrhagic bursal samples were more suitable compared to edematous bursal samples for the detection of IBDV antigen.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Abraham-Oyiguh J, Adewumi MO, Onoja AB, Suleiman I, Sulaiman LK, Ahmed SJ, Jagboro ST (2015). Seroprevalence of infectious bursal disease virus in local chickens in udu local government area of delta state, south east Nigeria. Journal of Immunoassay and Immunochemistry 36:398-404.

Aliev AS, Dzhavadov ED, Leont'eva MM (1990). Indirect haemagglutination test for infectious bursal disease of fowls. Veterinary Bulletin 60:47-67.

Aliyu HB, Sa'idu L, Jamilu A, Andamin AD, Akapavie SO (2016). Outbreaks of Virulent Infectious bursal disease in flocks of battery cage brooding system of commercial chickens. Journal of Veterinary Medicine 2016:8182160.

Allan GM, Mc Nulty MS, Connor TJ, Mc Cracken RM, Mc Ferran JB (1984). Rapid diagnosis of infectious bursal disease infection by immunofluorescence on clinical materials. Avian Pathology 13:419-427.

Butt TM, Rafique R, Rauf U, Khan MN, Nayyer A, Abbas Y, Farooq T (2015). Adaption of infectious bursal disease virus (ibdv) on chicken embryo-fibroblast culture. International Journal of Agricultural Science Research 4(1):021-023.

Chowdhury EH, Islam MR, Das PM, Dewan MK, Khan MSR (1996). Acute infectious bursal disease in chickens: pathological observation and virus isolation. Asian-Australasian Journal of Animal Sciences 9(4):465-469.

Daodu OB, Oludairo OO, Aiyedun JO, Ambali HM, Kadir RA, Daodu OC, Isaac Dayo Olorunshola ID, Adah AD (2018). Assessment of antibody assay methods in determination of prevalence of infectious bursal disease among local chickens and guinea fowls in Kwara state, North Central Nigeria. Veterinary World 11(8):1183-1187.

Dey S, Pathak DC, Ramamurthy N, Maity HK, Chellappa MM (2019). Infectious bursal disease virus in chickens: prevalence, impact, and management strategies. Veterinary Medicine: Research and Reports 10:85–97.

El-Samadony HA, Mekky HM, Mahgoub KM (2019). Molecular characterization of field isolates of Gumboro virus. Bioscience Research 16(1):171-182.

Escaffre O, Le Nouën C, Amelot M, Ambroggio X. Ogden KM, Guionie, O, Toquin D, Müller H, Islam MR, Eterradossi N (2013). Both genome segments contribute to the pathogenicity of very virulent infectious bursal disease virus. Journal of Virology 87: 2767-2780.

Garibyan L, Avashia N (2013). Polymerase Chain Reaction. Journal of Investigative Dermatology 133(3):1-4.

Hasan KMR, Ali MH, Siddique MP, Rahman MM, Islam MA (2010). Clinical and laboratory diagnoses of newcastle and infectious bursal diseases of chickens. Bangladesh Journal of Veterinary Medicine 8(2):131-140.

Hassan MK (2004). Very virulent infectious bursal disease virus in Egypt: epidemiology, isolation and immunogenicity of classic vaccine.

Veterinary Research Communications 28(4):347-356
.Hornbeck P (2017). Double-immunodiffusion assay for detecting specific antibodies (Ouchterlony). Current Protocols in Immunology 116:2.3.1-2.3.4.
Hussain I, Zahoo MA, Rasool MH, Shahid- Mahmood MM, Mansoor MK, Riaz MN (2003). Detection of Serum antibody levels against Infectious bursal disease (IBD) virus using indirect haemagglutination (IH) test in commercial broilers. International Journal of Poultry Science 2(6):442-445.
Jackwood DJ, Stoute ST (2012). Molecular evidence for a geographically restricted population of infectious bursal disease viruses. Avian Diseases 57:57-64.
Khan RSA, Sajid S, Habib M, Ali W, Shah MS, Sarfraz M (2017). History of Gumboro (infectious bursal disease) in Pakistan. Saudi Pharmaceutical Journal 25(4):453-459.
Khan RW, Khan FA, Farid K, Khan I, Tariq M, Sheikh F, Banga S, Banga S, Najeeb S, Lone B (2009). Prevalence of infectious bursal disease in broiler indistrict Peshawar. Journal of Agricultural and Biological Sciences 4:1-5.
Kralik P, Ricchi M (2017). A basic guide to real time PCR in microbial diagnostics: definitions, parameters, and everything. Frontiers in Microbiology 8:108.
Kusk M, Kebell S, Jorgensen PH, Handberg KJ (2005). Differentiation of five strains of infectious bursal disease virus: development of a strain specific multiplex PCR. Veterinary Microbiology 109:159-167.
Mahmood MS, Siddique M (2006). Comparative efficacy of RT-PCR, AGPT and reverse passive haemagglutination assay for the detection of infectious bursal disease virus in broilers. Pakistan Veterinary Journal 26(4):167-170.
Mawgod SA, Arafa AS, Hussein HA (2014). Molecular genotyping of the infectious bursal disease virus (IBDV) isolated from broiler flocks in Egypt. International Journal of Veterinary Science and Medicine 2:46-52.
Michel LO, Jackwood DJ (2017). Classification of infectious bursal disease virus into genogroups. Archives of Virology 162(12):3661-3670.
Nachimuthu K, Rai GD, Thangavelu A, Venkatesan RA (1995). Reverse passive haemagglutination test in the diagnosis of infectious bursal disease. Tropical Animal Health and Production 27:43-46.
Okwor EC, Eze DC, Okonkwo KE, Ibu JO (2011). Comparative evaluation of agar gel precipitation test (AGPT) and indirect haemagglutination test (IHA) for the detection of antibodies against infectious bursal disease (IBD) virus in village chickens. African Journal of Biotechnology 10(71):16024-16027.
Oluwayelu DO, Adebiyi AI, Olaniyan I, Ezewele P, Aina O (2014). Occurrence of Newcastle disease and infectious bursal disease virus antibodies in double-spurred francolins in Nigeria. Journal of Veterinary Medicine 2014: 106898.
Ouchterlony O (1948). In vitro method for testing the toxin-producing capacity of diphtheria bacteria. Acta Pathologica Microbiologica Scandinavica 25:186-191.
Ouchterlony O, Nilsson L-A (1986). Immunodiffusion and immunoelectrophoresis. In Handbook of Experimental Immunology, Vol. 1: Immunochemistry (D.M. Weir, L.A. Herzenberg,C. Blackwell, and L.A. Herzenberg, eds.) 32.1-32.50.
Rodriguez-Chavez IR, Rosenberger JK, Cloud SS, Pope CR (2002). Characterization of the antigenic, immunogenic, and pathogenic variation of infectious bursal disease virus due to propagation in different host systems (bursa, embryo, and cell culture). III. Pathogenicity. Avian Pathology 31(5):473-483. DOI: 10.1080/0307945021000005851.
Rauw F, Lambrecht B, Van den Berg T (2007). Pivotal role in the pathogenesis of ChIFNgamma and immunosuppression of infectious bursal disease. Avian Pathology 36(5):367–374.
Roy P, Raja A, Dhillon AS (2008). Counter immunoelectrophoresis for diagnosis of infectious bursal disease: comparative efficacy of three different electrophoresis buffers. Journal of Applied Poultry Research 17:116-120.
Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA et al. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350-1354.
Sali K (2019). Overview of methods used in the diagnosis of infectious bursal disease. Veterinary Medicine Open Journal 4(1):9-17.
Teshome M, Admassu TFB (2015). Infectious bursal disease (GUMBORO Disease) in chickens. British Journal of Poultry Sciences 4(1):22-28.
Van den Berg TP, Eterradossi N, Toquin D, Meulemans G (2000). Infectious bursal disease (Gumboro disease). Scientific and Technical Review of the Office International des Epizooties 19(2):527-543.
Vogel J, Yee C, Darling J (2012). Molecular biology. In: Bolognia J, Jorizzo J, Rapini R (eds) Dermatology, 3rd edn.Elsevier: Philadelphia, PA, pp. 65-67.
Wang A, Liu F, Wang Z, Jiang, X, Wang, W, Teng K, Xu J (2011). Pathological study of SPF chickens experimentally infected with a chinese IBDV strain BC6/85. Asian Journal of Animal and Veterinary Advances 6(1):36-50.
Zahoor MA, Abubakar M, Naim S, Khan QM, Arshed MJ (2010). Incidence and Molecular Characterization of Infectious Bursal Disease Virus in Commercial Broilers in Pakistan. International Journal for Agro Veterinary and Medical Sciences 4(3):75-80.
Zohair GAM, Amer MM, EL-shemy A, Bosila MA, Elbayoumi KM (2017). Diagnosis and molecular identification of virulent infectious bursal disease in naturally infected broiler chickens. International Journal of Pharmaceutical and Phytopharmacological Research 7(5):29-34.

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Microbiological and parasitological contamination of vegetables, water and soil in rural communities

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Microbiological and parasitological contamination of vegetables, water and soil in rural communities of a municipality in the state of Bahia, Brazil, was assessed. Samples of *Lactuca sativa,* L., *Coriandrum sativum* and *Solanum lycopersicum*, soil, and irrigation water were analyzed between August and October, 2015. Total coliforms (TC), *Escherichia coli*, molds and yeasts on vegetables, soil and water, heterotrophic bacteria in water and soil were counted. Parasitological analyses were performed by spontaneous sedimentation method and by Rugai technique for vegetables and soil and by direct and Faust exams for water. Physical and chemical analyses included pH, temperature, dissolved oxygen and turbidity. TC counts were higher in lettuce samples (mean 2.8 log CFU g^{-1}). *E. coli* counts did not comply with legislation in a lettuce sample with 3.3 log CFU g^{-1}. TC had the highest counts, with mean between 3.7 and 4.9 log CFU g^{-1} in soil samples. All water samples showed poor conditions and most samples were positive for at least one parasite. Due to high microbial density and several parasite types in most samples, results showed poor sanitary quality of vegetables with health risks for people. It is crucial to invest in educational activities for handlers and farmers so that a better vegetable quality could be offered to the population. More efficient monitoring is required by health authorities, requiring periodic assessments for parasites so that consumers may have a better life quality.

Keywords: Agricultural produce, food production, food hygiene, sanitary profiles.

INTRODUCTION

Eating habits of many Brazilians have been modified throughout the years. There was great interest in *in natura* food, especially vegetables, due to known and reported health benefits (Ministry of Health, 2014). However, according to their characteristics, vegetables are frequently eaten raw or with inadequate cleaning, featuring possible contamination by microorganisms, such as helminths, protozoa, bacteria, fungi and viruses. The former are more common due to their ability to survive in soil and water with eventual outbreaks of food-borne diseases (FBD), with great risks for humans (Coutinho et al., 2015).

The contamination of vegetables by bacteria, fungi and parasites may occur through several ways, especially by contact with water used in the irrigation of vegetables contaminated with human fecal material, soil with organic manure derived from fecal dejects or by the contamination of food handlers´ hands (Fernandes et al.,

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2015). Microorganisms may indicate pathogens and inadequate hygiene conditions in production, transport and storage (Abreu et al., 2010). Due to the interference of several factors in the contamination of vegetables and lack of studies on these factors as sources of contamination, current paper evaluates the microbiological and parasitological contamination of vegetables, water and soil in rural communities in a municipality of the state of Bahia, Brazil.

MATERIALS AND METHODS

Selection of vegetables

This research was characterized as a descriptive and analytical cross-sectional study. The selection of vegetables for current study was foregrounded by data retrieved from Family Budget Research by the Instituto Brasileiro de Geografia and Estatística which evaluated food consumption data of the Brazilian population. FBR data and local producers´ survey were the criteria employed to include food with the greatest consumption rates and which were produced by communities under analysis. Three vegetables were chosen, namely lettuce (*Lactuca sativa*) and tomatoes (*Solanum lycopersicum)*, respectively with consumption percentages 4.7 and 2.4%, and parsley (*Coriandrum sativum*). The latter was included because it was greatly used in crude salads, with a 10.6% consumption rate (IBGE, 2011).

Farms characteristics

Qualitative half-structured interviews were previously undertaken on topics agreed upon by farm owners or laborers. The questionnaire consisted of 17 questions divided into three topics: I Property identification (7 questions). II Production data (10 questions). Item I provided information about the presence of pets, existence of sanitary facilities, and destination of sewage and place where it performs the physiological needs. Item II provided information on the type and destination of the vegetables produced, the type of fertilizer and / or pesticide used in the production, origin and treatment of the water used for vegetable production and family consumption.

Producers who reported performing organic planting were chosen. Nine producers residing in four rural communities in the micro region of Santo Antonio de Jesus, Bahia, Brazil, participated. The survey was conducted in the communities Sapucaia 13°2'43"S 39°16'34"W, Cunha 12°57'36"S 39°17'11"W, São Bartolomeu 12°58'26.3"S 39°16'56.1"W and Onha 13°00'41.5"S 39°04'06.9"W. The vegetables were sent to different places: proper consumption, restaurants, supermarkets, town fairs, local community and the National School Meal Program (PNAE).

Collection of samples

Material was collected between August and October, 2015. Samples of three vegetables, a sample of the soil where the vegetables were cultivated and a sample of the water for irrigation were retrieved from each producer. Soil samples were taken from different points, forming a compound sample and collected superficially (10-20 cm) at the points where the plant species developed. Each soil sample consisted of five subsamples (Silva et al., 2007). We collected 63 samples, seven samples for each of nine producers: three vegetable samples, three soil samples and one water sample. Soil and vegetable leaves samples were performed by hand, in an aseptic way, in new plastic bags. Further, 1.5 L water samples were collected in new plastic recipients with lid. Samples were identified, refrigerated at 4°C and transported to the Multidisciplinary Complex for Studies and Research in Health (COMEPS) of the Center of Health Sciences (CCS) of the Universidade Federal do Recôncavo da Bahia (UFRB). There was a maximum 24 h between sample collection and start of analysis.

Microbiological analysis

Microbial populations of vegetables were estimated by Petrifilm^{TM} rapid count (3 M Company), from 25 g of each sample, with EC plates for TC and *Escherichia coli* and YM plates (AOAC 997.02) for molds and yeasts. Microbiological analysis of soil comprised TC, *E. coli*, molds and yeasts and heterotrophic bacteria counts. Populations of TC and *E. coli* were calculated with Petrifilm™ CE plates (3 M Company), whilst molds and yeasts were estimated with medium Martin with chloramphenicol (0.05%); heterotrophic bacteria were calculated by medium Trypticase Soy Agar (TSA), from 10 g of each sample, following Santos et al. (2007).

The water´s microbiological analysis comprised TC and *E. coli* by Readycult® Coliforms 100 rapid count (Merck KGaA 01), with estimated populations of heterotrophic bacteria by Petrifilm™ AQHC rapid count (3 M Company) and molds and yeasts with Petrifilm™ AQUA YM plates (AOAC 997.02), from 100 ml of each sample. Colonies were counted with colony count model CP600 Plus (Phoenix ®), calculating colony formation units (CFU) and log CFU g^{-1} of sample (APHA, 2001).

Parasitological analyses

Analytic methodologies for parasitological research in food and soil samples comprised spontaneous sedimentation method (Zeibig, 2014) and modified Rugai technique (Neves et al., 2016). The spontaneous sedimentation method comprised washing of vegetables in distilled water in new plastic bags, elimination of deteriorated and wrinkled parts, manual shaking for 15 min. Four grams of sampled soil were retrieved to which were added 200 ml of distilled water. The samples were filtered by a four-fold gauze and left for sedimentation for 24 h in a conic beaker (Zeibig, 2014). Modified Rugai technique consisted of involve a food sample or 4 g of soil in a four-fold gauze, submerged in a conic beaker with distilled water at 45°C for at least two hours. The gauze was then removed and the supernatant discarded. Further, 50 ml of the sediment was transferred into a sterile collector flask for the preparation of laminas for microscopic analysis (Neves et al., 2016).

Water was analyzed by direct and Faust tests (fluctuation in zinc sulfate). In the case of the direct test, the water sample was maintained under spontaneous sedimentation for 24 h at room temperature. Sediment was then collected by Pasteur pipette. Faust´s method comprised a 50 ml aliquot of the sediment in a centrifugal tube and centrifuged at 1,500 rpm. The sediment was then re-suspended with 10 ml of water for later centrifugation at 1,500 rpm. The sediment was mixed with 10 mL of zinc sulfate solution (density 1.18), and centrifuged again. The membrane formed at the liquid surface was removed with a double bacteriological spade and transferred to a lamina, stained with lugol, covered with a laminule and observed under a 400x optic microscope (Neves et al., 2016). Laminas were stained with lugol and directly observed under an optic microscope, magnified 100x and 400x, by counting the number of structures through the surface of the junction, lamina x laminule.

Physical and chemical analysis of water

Analysis for pH, temperature, dissolved oxygen (DO) and electric

conductivity were prepared on the field after water samples by multi-parameter gauge AK88 (AKSO); turbidity and color analyses were performed in the laboratory by multiprocessor bench test turbidity-meter T-1000 (LABOTEC™) (APHA, 2012).

Ethical aspects

Current study was submitted to the Committee for Ethics in Research with Human Beings of the Universidade Federal do Recôncavo da Bahia (UFRB) (CAAE: 04022312.0.0000.0056 – Authorization 1.167.637), following Resolution 466/12 of the Brazilian National Health Council. Members of participating families were invited to sign a Term of Free Consent to participate and authorize sample collection of vegetable produced, soil and irrigation water in the allotted sites as a criterion for inclusion in current research (Ministry of Health, 2012).

Statistical analysis

Data were analyzed by Fischer´s exact test, with Statistical Package for the Social Sciences (SPSS) 20 (IBM) at 5% significance level ($p<0.05$) to evaluate the relationship between variables and prevalence of microbiological and parasitological contamination.

RESULTS AND DISCUSSION

Microbial and parasitological contamination

Table 1 shows counts of total coliforms in vegetable samples, soil and irrigation water. TC counts in vegetables ranged between 1.0 and 5.0 log CFU g^{-1} of lettuce samples, between 1.5 and 5.3 log CFU g^{-1} of parsley samples and between <1 and 2.9 log CFU g^{-1} of tomato samples. Highest averages among the vegetables were detected in lettuce (2.8 log CFU g^{-1}). Highest count occurred in a parsley sample at 5.3 log CFU g^{-1} (Producer 9). The producer 7 had better TC counts for parsley (1.5 log CFU g^{-1}) and tomato samples (<1 log CFU g^{-1}) and soils samples near them (3.3 and 4.1 log CFU g^{-1} respectively). The producer 6 had the worst CT counts for parsley samples (4.7 log CFU g^{-1}) and parsley (5.0 log CFU g^{-1}) and tomato soil.(6.0 log CFU g^{-1}) near them.

Different results were reported in a study by Rincón et al. (2010) in Maracaibo, Venezuela, with parsley being the vegetable with the highest average contamination by TC when compared to other vegetables analyzed. Although Brazilian legislation does not establish any limits for TC, the presence of such microorganisms in the food under analysis is relevant and indicates poor hygiene practice during processing (Silva et al., 2016). Soil samples showed mean TC counts, between 3.7 and 4.9 log CFU g^{-1}.

Soil samples from tomato plantation had the highest contamination rates by TC, averaging 4.9 log CFU g^{-1}. Results could be a consequence of defecating directly in the soil, a common practice by rural producers analyzed, the free roaming of domestic animals and the use of manure without due processing (Coutinho et al., 2015).

TC was detected in all water samples. In fact, it failed to comply to the Ministry of Health´s Resolution 2914/2011 which establishes that one of the items for drinkable water is the absence of TC in 100 ml of the sample (Ministry of Health, 2011). Average *E. coli* counts in vegetables ranged between <1.0 and 1.1 log CFU g^{-1}. Lettuce showed the highest contamination rate, with mean count of 1.1 log CFU g^{-1}, and tomato by the lowest contamination of the bacterium, with populations <1.0 log CFU g^{-1} (Table 2). Producers 1 and 2 had the worst TC counts. The highest counts from Producer 1 were from soil samples close to parsley (3.6 log CFU g^{-1}) and tomato cultivation (3.0 log CFU g^{-1}) and the highest counts from Producer 2 were from lettuce (3.3 log CFU g^{-1}) and the soil near the cultivation of this vegetable (3.6 log CFU g^{-1}).

According to parameters by resolution of the collegiate board (RDC) 12/2001 of the Brazilian Agency of Sanitary Care (Anvisa, 2001), one lettuce sample (Producer 2) was contaminated above the limits by *E. coli* (2.0 log CFU g^{-1}) and thus not good for consumption (Table 2). Similarly, Silva et al. (2016) evaluated the quality of water used in the irrigation of five vegetable gardens and in food irrigated in a swampy area in the municipality of Caruaru, in the Agreste region of the state of Pernambuco, Brazil. The authors detected high contamination rates by TC and *E. coli*. Lettuce and parsley among the vegetables analyzed had the greatest contamination rates. In samples of irrigation water, they were all positive for TC and *E. coli.*

According to Yang et al. (2012), lettuce contamination by *E. coli* could occur anytime between the farm and the consumer´s table, predominantly by contact with fecal material due to the use of manure in the plantation and/or bad hygiene practices at home. In fact, two samples of soil with lettuce (Producers 2 and 3), a sample of soil with parsley and a sample of soil with tomato (Producer 1) had *E. coli* populations above 3.0 log CFU g^{-1}. According to Naganandhini et al. (2015), partially decomposed manure, slurry, wastes from abattoirs and human sewerage could be the source of contamination of arable agricultural land since certain species of *E. coli* could survive in manure based on animal excrement for long periods (between 25 to over 365 days). They could infect humans even with low cell load (10-500/g).

In the case of water samples, *E. coli* was detected in 88.9% of samples (Table 2), which was inadequate according to the Ministry of Health´s Resolution 2914 of December 12, 2011, which determined the absence of *E. coli* in 100 ml of the samples. According to Araújo et al. (2015) this contamination could be due to domestic sewage.

One should highlight that samples of tomato in current study had low TC and *E. coli* counts, perhaps due to the anatomical characteristics of the tomato. Similarly, Arbos

Table 1. Counts of total coliforms in vegetables, soil and irrigation water of producers in rural communities.

	Vegetables[1]			Soil[1]			Water[2]
Producer	**Lettuce**	**Parsley**	**Tomato**	**Soil lettuce**	**Soil parsley**	**Soil tomato**	
1	1.3	2.6	1.5	3.9	4.7	4.7	P
2	4.0	2.0	2.7	4.7	4.0	5.0	P
3	5.0	2.5	2.9	4.4	3.3	4.8	P
4	3.0	1.7	2.1	3.9	3.6	5.2	P
5	2.4	2.8	<1	4.0	3.4	4.9	P
6	3.8	4.7	<1	4.0	5.0	6.0	P
7	1.0	1.5	<1	3.7	3.3	4.1	P
8	3.9	1.8	1.6	3.3	4.3	5.2	P
9	1.0	5.3	<1	3.0	2.5	4.6	P
Means	2.8	2.7	1.6	3.8	3.7	4.9	-
SD	1.3	1.2	0.7	0.4	0.7	0.3	-

[1] log CFU g-1; [2] Presence (P); [3] Standard Deviation (SD).

Table 2. *E. coli* counts in vegetables, soil and irrigation water of producers in the rural communities.

Producer	Vegetables[1]			Soil[1]			Water[2]
	Lettuce	**Parsley**	**Tomato**	**Soil Lettuce**	**Soil Parsley**	**Soil Tomato**	
1	<1	<1	<1	<1	3.6	3.0	P
2	3.3	<1	<1	3.6	<1	<1	A
3	<1	1.5	<1	3.0	<1	<1	P
4	<1	<1	<1	<1	<1	<1	P
5	<1	1.0	<1	<1	<1	<1	P
6	<1	<1	<1	<1	<1	<1	P
7	<1	1.0	<1	<1	<1	<1	P
8	<1	1.0	<1	<1	<1	<1	P
9	<1	1.0	<1	<1	<1	<1	P
Means	1.1	1.0	<1	1.4	1.2	1.1	-
SD	0.7	0.1	0.7	0.9	0.9	0.6	-

[1] log CFU g-1; [2] Presence (P) Absence (A); [3] Standard Deviation (SD).

et al. (2010) also reported low contamination in tomato when compared to the other vegetables under analysis. All vegetable samples showed MY with mean counts between 1.6 and 5.0 log CFU/ g^{-1}. MY counts in soil samples were high, varying between 4.7 and 4.9 log CFU g^{-1}. Only one sample failed to show MY colony growth, whilst count samples ranged between <1.0 and 5.0 log CFU g^{-1}. Producer 2 had the worst MY counts for lettuce (5.5 log CFU g^{-1}) and parsley samples (5.8 log CFU g^{-1}) (Table 3).

Santos et al. (2010) reported that although MY are important indicators of food contamination, maximum counting limits were not predicted for most foods (Santos et al., 2010). Current study also estimated counts of heterotrophic bacteria (HB) in samples of soil of vegetable gardens. Counts ranged between 6.1 and 6.4 log CFU g^{-1}. Studies by Borges Filho and Machado (2013) revealed HB between <1.0 and 10 log CFU g^{-1} in soils after the addition of organic manure in Belo Horizonte, Brazil, considered high by the authors. Similarly, bacteria counts were high and may be a source of contamination to cultivated vegetables. HB counts were estimated in irrigation water samples. Sample from Producer 7 had the lowest HB count, whereas sample from Producer 5 proved to be the highest, respectively 1.4 and 5.5 log CFU g^{-1}. Sample from Producer 7 was retrieved from an artesian well and stored in a reservoir on the top of the house. The sample of Producer 5 was different even though it was retrieved from the public water supply and stored in an open reservoir on the floor. Only two water samples (Producers 7 and 8) had had populations within the maximum limit allowed

Table 3. Molds and yeast counts in vegetable, soil and irrigation water of producers in the rural communities.

Producer	Vegetables[1]			Soil[1]			Water[1]
	Lettuce	Parsely	Tomato	Soil Lettuce	Soil Parsely	Soil tomato	
1	5.4	5.1	4.5	4.7	4.7	5.1	3.0
2	5.5	5.8	2.1	4.7	5.0	4.6	2.0
3	4.6	4.6	4.5	5.1	4.8	4.9	2.0
4	4.4	4.9	5.0	4.0	5.2	4.7	2.0
5	4.6	4.8	4.3	5.0	4.9	5.0	4.0
6	5.1	5.1	4.0	4.6	6.0	4.6	0
7	5.5	5.3	4.0	4.6	4.1	5.0	5.0
8	4.9	5.3	5.2	4.8	5.2	4.7	4.0
9	3.9	3.9	2.8	4.9	4.6	5.7	1.0
Means	4.8	5.0	4.0	4.7	4.9	4.9	2.5
SD	0.5	0.5	0.7	0.2	0.4	4.3	1.4

[1] CFUv; [3] Standard Deviation (SD).

(respectively 1.4 and 1.8 log CFU g^{-1}), or rather 2.7 log CFU ml^{-1}. Consequently, 78% of samples did not comply with current legislation (Ministry of Health, 2011). According to Araújo et al. (2015), HB counts are dangerous at high concentrations, although counts are employed as a complementary parameter to thermotolerant coliforms such as *E. coli*.

At least one parasite species was extant in most samples analyzed (79.3%). The 2014 RDC Resolution of ANVISA determined the absence of parasites, at any phase of development, in food (Table 4). According to the above legislation, 77.8% of vegetable garden samples were not fit for human consumption (ANVISA, 2014). Lettuces and parsley had the highest number of positive samples. Similarly, lettuces in studies on vegetables had the highest parasite contamination (Constantin et al., 2013; Coutinho et al., 2015; Brauer et al., 2016). Since lettuce and parsley grow close to the soil, they are more prone to contamination by parasites. Protozoa cysts and helminth eggs survive for long periods in the soil and are still viable at the moment of intake of vegetables (Brauer et al., 2016).

Five tomato samples (55%) were contaminated by helminths, contrasting reports by Abreu et al. (2010) who highlighted that the distance of the tomatoes above the soil makes contamination by parasites difficult. Parasites which are most frequently found in vegetable samples were ciliated protozoa cysts (81%), cysts of other protozoa (44.4%), larvae of nematode helminths (18.5%), adults of nematode helminths (14.8%), adult mites (14.8%), cysts of *Endolimax nana* (14,8%), eggs of *Ancylostoma* spp (3,7%) and eggs of trematode helminths (3.7%). Soil samples contained ciliated protozoa cysts (51.8%), cysts of other protozoa (40.7%), larvae of nematode helminths (37%), adults of nematode helminths (22.2%), eggs of nematode helminths (7.4%), eggs of *Ancylostoma* spp (3.7%), eggs of *Trichuris* spp. (3.7%).

Since no species classification key is extant in the laboratory, it was not possible to identify the species of several parasites in the material under analysis. Although most species were not pathogenic, they nevertheless indicate the poor sanitary conditions in vegetable breeding. It is also highly relevant to detect helminths such as *Ancylostoma* spp, not merely because of their high prevalence but due to the diversity of their clinical manifestations in the hosts (Mesquita et al., 2015). Prevalence of protozoa was reported in all samples, when compared to helminths. Parasites may be vectors in several ways and, due to their great adaptability and resistance, they could coexist in highly uncommon media (Terto et al., 2014).

As a rule, parasite contamination occurred more in vegetable than in soil samples. This result was due to contamination during handling by infected handlers or through contact with fecal dejects vectored by animals such as fowl, flies, mice and insects. Since the latter stay on faeces in the open air and then fly onto food, they were important mechanical vectors of parasites (Bauer et al., 2016). Current legislation has not provided maximum and minimum rates for water temperature for human consumption. However, temperature between 24 and 32.5°C of water samples (Table 5) was prone to develop TC groups that survive temperatures up to 37°C (Silva et al., 2016).

Further, pH is linked to bacterial multiplication since most the best pH for most bacteria oscillates between 6.5 and 7.5. In other words, pH past neutrality is related to the introduction of organic matter and home effluents. Current study reveals that four samples were inadequate, featuring pH rates below that recommended (6.0- 9.5) (Ministry of Health, 2011).

In the case of turbidity, the sample of Producer 1 only was not satisfactory due to non-compliance with standard

Table 4. Parasites in samples of vegetables, soil and irrigation water in rural communities.

Samples	Helminths		Protozoa		Bi-parasitism		Absence	
	n	%	n	%	n	%	n	%
Vegetables								
Lettuce	-	-	6	67	3	33	-	-
Parsley	-	-	6	67	3	33	-	-
Tomato	5	55	1	11	1	11	2	22
Soil								
Lettuce	-	-	2	22	6	67	1	11
Parsley	1	11	2	22	4	44	2	22
Tomato	2	22	3	33	2	22	2	22
Water	-	-	2	22	1	11	6	67

[1] number (n).

Table 5. Physical and chemical parameters of irrigation water used in the production of vegetables of producers in rural communities.

Producer	Physical and chemical parameters			
	Temperature. (Cº)	pH	DO (mg L-1)	Turbidity. (NTU)
1	25.2	5.8	7.0	7.28
2	25.5	4.95	5.0	0.19
3	24.2	4.7	4.5	1.25
4	27.0	5.94	6.0	0.02
5	24.0	6.5	6.6	4.43
6	32.5	6.41	4.0	3.86
7	27.9	6.46	8.7	2.14
8	25.9	6.5	9.9	3.13
9	30.5	6.88	7.2	1.34

[1]Dissolved Oxygen (DO); [2] Nephelometric Turbidity Unit (NTU).

of up to 5.0 NTU (Ministry of Health, 2011). So that the water´s microbiological quality may be guaranteed, the turbidity pattern should be monitored. In fact, several studies have shown that pathogens, such as *Cryptosporidium* spp., has been associated with turbidity. In fact, the greater the water´s turbidity rate, the greater is the possibility of detecting parasites (Daneluz; Tessaro, 2015).

Oxygen is essential for aerobic organisms, DO amounts assess water conditions, during the aerobic equilibrium of the water´s organic matter, decomposition-causing bacteria use oxygen in their respiratory processes and may decrease it in the medium. The greater the charge of organic matter, the greater is the number of decomposition-causing microorganisms and, consequently, greater oxygen consumption (Araújo et al., 2015). In current study, DO rates ranged between 4.0 and 9.9 mg/dL, even though Resolution 2914/2011 have not established DO rates in water for human consumption.

Allende et al. (2015) revealed that irrigation water is the main source of contamination in vegetables. Water contamination by human faeces originates from public or home sewage discharges and from direct release of fecal material on the water surface by domestic or wild animals. Since many people failed to treat water adequately, a possible transmission chain was established. Health risks exist when water is taken or food washed by contaminated water is ingested (Fernandes et al., 2015).

Fischer´s test was employed to assess the relationship between contamination of vegetables, soil and water irrigation. No statistical relationship between the variables was detected. Although a statistical co-relationship between variables was not established, the results of the analysis were highly relevant due to the great importance that contamination of vegetables represented for public health. Good Agricultural Practices (GAP) during the cultivation of vegetables is basic to prevent contamination and protect consumers´ health.

However, proper washing of vegetables by the

population prior to consumption is mandatory for healthy food, with a reduction in microbial counts. The Brazilian Agency for Sanitary Care (ANVISA) recommends the use of sodium hypochlorite 200 ppm for 10 min as a sanitizing method so that vegetables may be safely consumed (ANVISA, 2004).

Farms characteristics

Nine producers were interviewed, of which six were between 30 and 55 years old and three over 55 years old. The survey showed that seven were males and only two were females. Also, seven of the producers had incomplete elementary school and two did not have any formal education. Avendaño-Reyes et al. (2019) in a study conducted with dairy farmers in Mexico found that most farmers were also males (84 and 20%) had no formal education, but 40% had studied at elementary school and the average age of farmers was over 56 years, unlike our own findings.

All interviewed people cultivated planted the vegetables in open fields and called themselves producers of organic vegetables. They informed that they used cattle or goat manure and food wastes, although none had an organic producer certificate issued by the Ministry of Agriculture, Stockbreeding and Supply (MAPA). According to MAPA producers selling organic products should have a certificate issued by MAPA or they should organize in groups for direct commercialization without any certificate. In this case, the producer cannot sell to third parties but only in town fairs (or directly to consumers) and in purchases by the government (Brazilian Program for School Food) and for the National Supply Company (CONAB). Producer 4, for instance, was not authorized to sell organic vegetables but they were sold to restaurants and supermarkets (Mapa, 2003).

When the hygiene and sanitary conditions of the vegetable garden area with regard to domestic animal breeding were evaluated, it may be seen that all producers had domestic animals, such as dogs, cats and fowls, which freely roamed around the vegetable gardens. Only 66.6% of homes had any sanitary installations and used septic cesspits for sewage. The other producers with no sanitary installations excreted directly in the soil. Other residents (55.5%), even with sanitary devices in their homes, preferred to do the same.

According to Jesus et al. (2013), bad sanitary conditions contributed towards the dissemination of intestine parasitosis in rural and urban areas. Constantin et al. (2013) insisted that performing physiological needs directly in the soil was a common habit by rural inhabitants, implying inadequate disposal of human dejects. It increased contamination risks in the field. Further, the roaming of animals within or close to the vegetable gardens could contaminate vegetables by pathogens in their feces.

Eight producers (88.9%) used animal manure and only one (11.1%) reported the use of vegetable wastes and ashes and the producers did not practice composting. According to Sediyama (2014), animal manure should be processed for composting to decrease microbial load. In fact, when organic manure was treated or stored inadequately, it could contain pathogens and contaminate the vegetables. In fact, it favored the survival of helminth eggs and protozoa cysts in the medium up to the intake of the vegetables. The cistern was the means of water supply for irrigation by 55.5% of participants. Only Producer 5 employed water from the water supply system, even though the water was inadequately stored within an open tank. Unhealthy conditions of irrigation water were the rule in 88.9% of the vegetable gardens.

Conclusion

Due to high microbial density and the detection of parasites in most samples, one may say that health quality in the production of vegetables is not satisfactory possibly due to improper use of partially decomposed fertilizers, failure to treat water used for irrigation, contact of vegetables with feces waste, improper handling and bad home hygiene habits and is a risk to human health. It is crucial to invest in educational activities for handlers and farmers so that a better vegetable quality could be offered to the population. More efficient monitoring is required by health authorities, requiring periodic assessments for parasites so that consumers may have a better life quality.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Abreu IMDO, Junqueira AMR, Peixoto JR, Oliveira SAD (2010). Microbiological quality and productivity of lettuce under chemical and organic fertilization. Food Science and Technology 30(1):108-118.

Allende A, Monaghan J (2015). Irrigation Water Quality for Leafy Crops: A Perspective of Risks and Potential Solutions. International Journal of Environmental Research and Public Health 12(7):7457-7477.

American Public Health Association (APHA) (2001). Compendium of methods for the microbiological examination of foods. 4th edition. Washington, USA.

American Public Health Association (APHA) (2012). Standard methods for the examination of water and wastewater. 22nd edition. Washington, USA.

Araujo FV, Vieira L, Jayme MMA, Nunes MC, Cortês M (2015). Quality

evaluation of the water used for irrigation in the Córrego Sujo basin, Teresópolis, RJ. Caderno de Saúde Coletiva, Rio de Janeiro 23(4): 380-385.
Arbos KA, Freitas RJS, Stertz SC, Carvalho LA (2010). Organic vegetables safety: sanitary and nutritional aspects. Ciência e Tecnologia de Alimentos, Campinas 30(1): 215-220.
Avendaño-Reyes, L, Robinson, PH, Hernández-Rivera, JA, Correa-Calderón, A, López-López, Á, Mellado, M, Macías-Cruz, U (2019). Characterization of small-scale dairy farms and its relation to water use efficiency in the Mexicali Valley, Mexico. Tropical animal health and production 1-8.
Borges Filho ELB, Machado EC (2013). Evaluation of microbial soil and vegetables after the addition of organic fertilizers: a case study of the vegetable-garden. Revista e-Scientia, Belo Horizonte 6(1): 8-15.
Brauer AMNW, Silva JC, Souza MMAA (2016). Enteroparasites distribution in vegetables of São Mateus food trade, Espírito Santo, Brazil. Natureza on line 14(1): 55-060. Available at: http://www.naturezaonline.com.br/natureza/conteudo/pdf/NOL20151101.pdf
Brazil, Brazilian Agency of Sanitary Care, ANVISANational Health Council (2004). Resolution nº 216, 15 of September of 2004. Brasília, Brazil. Available at: http://portal.anvisa.gov.br/documents/33916/388704/RESOLU%25C3%2587%25C3%2583.
Brazil, Ministry of Agriculture, Livestock and Supply, MAPA, Brazil (2003). Ordinance Nº 10.831, 23 of December of 2003. Brasília, Brazil. Available at: http://www.planalto.gov.br/ccivil_03/leis/2003/L10.831.html.
Brazil, Ministry of Health, Brazil (2011). http://site.sabesp.com.br/site/uploads/file/asabesp_doctos/PortariaMS291412122011.pdfOrdinance Nº 2.914, 12 of December of 2011. Brasília, Brazil.
Brazilian Agency of Sanitary Care, ANVISA (2001). Resolution nº 12, 02 of January of 2001. Brasília, Brazil. Available at: http://portal.anvisa.gov.br/documents/33880/2568070/RDC_12_2001.pdf/15ffddf6-3767-4527-bfac-740a0400829b.
Brazilian Agency of Sanitary Care, ANVISA (2014). Resolution nº 14, 28 of March of 2014. Brasília, Brazil.
Constantin BS, Gelatti LC, Santos O (2013). Avaliação da contaminação parasitológica em alfaces: um estudo no sul do Brasil. Revista Fasem Ciências 2(1):9-22.
Coutinho MGS, Ferreira CS, Neves AM, Alves FRL, Souza FFP, Fontenelle ROS (2015). Avaliação microbiológica e parasitológica de alfaces (*Lactuca sativa*) comercializadas em feiras livres no município de Sobral – CE. Revista da Universidade Vale do Rio Verde, Três Corações 13 (2):388-397.
Daneluz D, Tessaro D (2015). Physico-chemical and microbiological standards of water springs and shallow wells on rural properties in the southwest region of Paraná. Arquivos do Instituto Biológico, São Paulo 82:1-5.
Fernandes NS, Guimarães HR, Amorim ACS, Reis MB (2015). Parasitological assessment of vegetables: from the kitchen garden to the consumer. Revista Saúde e Pesquisa 8(2):255-265.
IBGE (2011). Brazilian Institute of Geography and Statistics. Household Budget Survey 2008-2009: analysis of food consumption in Brazil / IBGE, Work and Income Coordination. Rio de Janeiro: IBGE150 P. Available at: http://biblioteca.ibge.gov.br/visualizacao/livros/liv50063.pdf.
Jesus JS, Rubens AOM, Rosemary FA, Margarete S, Mendonça G, Flávio HFB, Silvia MMF (2013). Enteroparasitosis prevalence among farmers fair producer of rural neighborhood buritizal, Macapá, Amapá, Brazil. Revista de Biologia e Ciências da Terra 13(2):74-78.
Mesquita DR, Silva JP, Monte NDP, Sousa RLT, Silva RVS, Oliveira SS, Leal ARS, Freire SM (2015). Ocurrence of parasites on curly lettuce (*Lactuca sativa* L.) cultured in community gardens in Teresina, Piauí, Brazil. Revista Patologia Tropical 44(1):67-76.
Ministry of Health, Brazil Brazil, National Health Council (2012). Resolution nº 466, Brazil.
Ministry of Health. Secretariat of Health Care. Department of Primary Care, Brazil (2014). Ministry of Health. Secretariat of Health Care. Department of Primary Care. Food guide for the Brazilian population. 2nd. Brasília: Ministry of Health, 2014. Available at: http://bvsms.saude.gov.br/bvs/publicacoes/guia_alimentar_populacao_brasileira_2ed.pdf.
Naganandhini S, John Kennedy Z, Uyttendaele M, Balachandar D (2015). Persistence of Pathogenic and Non-Pathogenic *Escherichia coli* Strains in Various Tropical Agricultural Soils of India. Journal PLOS ONE 10(6).
Neves DP, Melo AL, Linardi PM, Vitor RWA (2016). Human Parasitology. 13rd edition. São Paulo: Atheneu, Brazil.
Rincón VG, Ginestre M, Romero S, Castellano M, Ívila Y (2010). Microbiological Quality and Enteropathogenic Bacteria in Leaf Vegetables Kasmera 3238(2):97-105.
Santos LC, Antoniolli ZI, Leal LT, Lupatini M (2007). A População de bactérias e fungos no solo contaminado com cobre nas Minas do Camaquã, RS, Brasil. Ciência e Natura 29(4):105-114.
Santos TBA, Silva N, Junqueira VCA, Pereira JL (2010). Microbiological quality evaluation minimally processed vegetables. Brazilian Journal of Food Technology, Campinas 13(2):141-146.
Sediyama MAN, Santos IC, Lima PC (2014). Growing vegetables in the organic system. Revista Ceres, Viçosa 61:829-837.
Silva AFS, Lima CAD, Queiroz JJF, Jácome PRDLA, Jácome Júnior AT (2016). Bacteriological analysis of horticultural irrigation water. Revista Ambiente Água 11(2):428-438.
Silva N, Junqueira VCA, Silveira NFA (2007). Manual of methods of microbiological analysis of foods. Manual de Métodos de Análise Microbiológica de Alimentos, 3rd edn., Varela, São Paulo. Terto WDS, Oliveira RG, Lima MM (2014). Parasitological evaluation in lettuces (*Lactuca sativa* L.) marketed in Serra Talhada, Pernambuco, Brazil. Vigilância Sanitária em Debate 2(3):51-57.
Yang Y (2012). Assessment of *Escherichia coli* O157:H7 transference from soil to iceberg lettuce via a contaminated field coringnhar vesting knife. International Journal Food Microbiology 153(3):345–350.
Neves DP, Melo AL, Linardi PM, Vitor RWA (2016). Human Parasitology. 13rd edition. São Paulo: Atheneu, Brazil.
Zeibig EA (2014). Clinical parasitology: a clinical and laboratory approach. 2rd edition. Rio de Janeiro: Elsevier, Brazil. https://www.elsevier.com/books/clinical-parasitology/zeibig/978-1-4557-5049-8

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Studies on optimization of L-glutaminase production under submerged fermentation from marine *Bacillus subtilis* JK-79

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In the current study, a marine strain *Bacillus subtilis* JK-79 capable of producing L-glutaminase enzyme, was analyzed for maximal L-glutaminase production. The Plackett-Burman design (PBD) was applied to determine the significant variables. Optimum conditions of the significant variables on L-glutaminase production were determined by the Central Composite Design (CCD) of response surface methodology. Maximum L-glutaminase production of 691.27 U/ml under flask condition was obtained at the predicted optimal values of pH 6.9, fructose 2.1% (w/v), yeast extract 1.25% (w/v), and glutamine 2.47% (w/v). Statistical optimization has enhanced L-glutaminase enzyme production upto 3.48 fold when compared with the basal SWG medium. The results of this study revealed that marine *B. subtilis* JK-79 could be a promising source for L-glutaminase production.

Key words: *Bacillus subtilis* JK-79, L-gltaminase, response surface methodology, submerged fermentation.

INTRODUCTION

Leukemia leads to the lympo-proliferative disorders in particular acute lymphoblastic leukemia (ALL). According to facts and statistics, Leukemia and Lymphomo Society (2018), 60,300 people are expected to be diagnosed with leukemia in United States and 24,370 people are expected to die from leukemia (14,270 males and 10,100 females). In 2010 to 2014, leukemia was the sixth most common cause of cancer deaths in both men and women (American Cancer Society's Cancer Facts and Figures, 2018). The treatment of ALL is very complex with drugs and there is reluctance for use in children and adults as it results in severe toxic reactions (Douer, 2008; Noura et al., 2014). In recent years, cancer therapy is highly relied on enzymes as they are low molecular weight protein molecules specific in their action and have less or no toxic effects. The enzymatic approach was reported to be more promising cancer therapy, due to the discovery of several enzymes with effective anti-cancer activity on various types of cancer (Vellard, 2003; Pandian et al., 2014).

L-glutaminase an amidohydrolase enzyme (E.C 3.5.1.2) has been found to be promising in the treatment

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of ALL (Souba, 1993; Vijayan et al., 2017). Cancer cells, especially ALL cells cannot synthesize L-glutamine, as they lack properly functioning glutamine biosynthetic machinery and therefore require large amount of L-glutamine for their rapid growth. These cells depend on the exogenous supply of L-glutamine for their survival and rapid cell division. Hence, the use of L-glutaminase deprives the tumor cells from L-glutamine and causes selective death of L-glutamine dependant tumor cells. Thus, it can act as a possible candidate for enzyme therapy (Hensley et al., 2013; El-Ghonemy, 2014). In recent years, L-glutaminase in combination with or as an alternative to L-asparginase could be used as in enzyme therapy for cancer particularly leukemia (Sabu, 2003).

The production of L-glutaminase by bacteria has attracted great attention owing to their cost effective and eco-friendly nature (Chandrasekaran, 1996; Unissa et al., 2014). Microbial L-glutaminase can be produced by both submerged and solid state fermentation. The improvement in production of L-glutaminase by a microbial strain can be achieved by determining the optimum physico-chemical parameters. Selection of appropriate carbon and nitrogen source is one of the most critical stages in the development of an efficient and economical production process. The methodologies used for screening of the components fall into two major categories: classical and statistical. The screening of various cultural parameters by classical method involves changing one independent variable (component of medium, parameter) at a time while fixing all other at certain level. This single dimensional search is simple, easy and useful technique to evaluate the individual effects of the media components and process conditions (Iyer and Singhal, 2008, 2009, 2010a, b) and has been commonly used for optimization.

However, the interaction between components influencing the production of a particular metabolite cannot be studied by varying one factor at a time (OFAT). Moreover, it often does not allow determination of actual optimum level of different components for a particular fermentation. The statistical methods provide an alternative solution to optimize a particular process by considering mutual interactions among the variables and give an estimate of the combined effect of these variables on the final result. Moreover, statistical methods are less laborious and rapid; thus are preferred over conventional OFAT approach for process optimization (Montgomery, 2000). Various statistical designs ranging from 2-factorial to multi-factorial are available for optimization of process parameters (Montgomery, 2000; Myers et al., 2009).

There has been much work reported on the effect of growth medium on the production of L-glutaminase by different marine bacteria. Various carbon and nitrogen sources were found to improve the production of L-glutaminase. Extracellular L-glutaminase producing *Beauveria* species BTMF S10 was isolated from marine sediment (Keerthi et al., 1999) and the yield was improved when the medium was supplemented with 1% yeast extract and sorbitol, 9% sodium chloride and 0.2% methionine at pH 9, 27°C. Optimum production of L-glutaminase from *Streptomyces rimosus* was observed at 27°C, pH 9 and glucose and malt extract as carbon and nitrogen sources, respectively (Sivakumar et al., 2006). Iyer and Singhal (2008, 2009), observed that the carbon and nitrogen sources for L-glutaminase production varied with the organisms. Supplementation of sucrose and yeast extract as carbon and nitrogen source improved L-glutaminase production by *Zygosaccharomyces rouxii* while higher L-glutaminase production was noticed in *Providencia* species with glucose and urea as carbon and nitrogen source, respectively. Recently, there are several reports which confirm the fact that the carbon and nitrogen sources significantly affect the production of L-glutaminase by microorganisms (Sathish and Prakasham, 2013; Krishnakumar et al., 2011; Pandian et al., 2014; Jesuraj et al., 2017).

In the present investigation, the potent marine strain *Bacillus subtilis* JK-79 was evaluated for maximal production of the L-glutaminase enzyme. In this context, different production media, carbon and nitrogen sources were tested for the highest yield of the L-glutaminase production.

MATERIALS AND METHODS

Microorganism and culture maintenance

The strain *B. subtilis* JK-79 (KC492745) used in this study was isolated from marine soil collected from Parangipettai costal area (Kiruthika and Saraswathy, 2014).

L-Glutaminase assay and protein estimation

L-glutaminase was assayed by the method of Imada et al. (1973). One international unit of L-glutaminase was defined as the amount of enzyme that liberates 1 µmol of ammonia under optimal conditions. The enzyme yield was expressed as Units/ml (U/ml of culture supernatant). Protein content in the crude enzyme source was estimated by Lowry's method (Lowry et al., 1951) using bovine serum albumin as the standard and the values were expressed as mg/ml.

Optimization by statistical design

Identifying significant variables by Plackett-Burman Design (PBD)

The Plackett-Burman Design (PBD; Mini Tab, trial version 17) was used to identify the significant variables affecting the L-glutaminase production. A total of eight factors such as pH (A), temperature (B), agitation speed (C), inoculum size (D), incubation time (E), carbon source (F), nitrogen source (G), and glutamine level (H), were considered and their high and low levels are shown in Table 1.

The responses from the 12 individual experiments were utilized for generating regression co-efficient values and the significant variables were further optimization by central composite design (CCD).

Table 1. High and low levels of variables used in PBD.

S/N	Coded variable	Factor	Low level (-1)	High level (+1)
1	A	pH	5	9
2	B	Temperature	25°C	45°C
3	C	Agitation speed	50 rpm	200 rpm
4	D	Incubation time	6 h	24 h
5	E	Inoculum size	1% (v/v)	7% (v/v)
6	F	Carbon source (fructose)	1% (w/v)	5% (w/v)
7	G	Nitrogen source (Yeast extract)	1% (w/v)	5% (w/v)
8	H	Glutamine	1% (w/v)	5% (w/v)

Table 2. PB experimental design for evaluating factors influencing L-glutaminase production by *Bacillus subtilis* JK-79.

Run order	A (pH)	B (Temperature)	C (Agitation speed)	D (Incubation time)	E (Inoculum size)	F (Carbon source)	G (Nitrogen source)	H (Glutamine)	L-glutaminase activity (U/ml)	Protein concentration (mg/ml)
1	-1	-1	1	1	1	-1	1	1	566.67	11.4
2	1	-1	1	1	-1	1	-1	-1	233.33	4.11
3	1	1	1	-1	1	1	-1	1	450	9.12
4	-1	1	-1	-1	-1	1	1	1	666.67	13.4
5	-1	-1	-1	1	1	1	-1	1	466.67	10
6	-1	1	1	1	-1	1	1	-1	412.22	8.78
7	1	1	-1	1	1	-1	1	-1	192.33	4
8	1	-1	-1	-1	1	1	1	-1	383.33	8.82
9	1	-1	1	-1	-1	-1	1	1	366.67	8.1
10	-1	-1	-1	-1	-1	-1	-1	-1	150	3.23
11	1	1	-1	1	-1	-1	-1	1	175	3.86
12	-1	1	1	-1	1	-1	-1	-1	183.33	3.98

Response surface methodology

The significant variables screened through PBD technique were subjected to CCD Software Design Expert Release 9 (Stat-Ease INC. Minneapolis MN, USA), a popular second order experimental design for developing sequential experimentation and predicting the levels of factors to get an optimal response through regression analysis. The effects of four independent variables *viz.* pH, carbon source, nitrogen source and glutamine on the production of L-glutaminase were studied at five different levels (-2, -1, 0, 1, 2). A full factorial central composite design was performed to build a total of 30 experiments, having $2^4 = 16$ cube points and 6 centre points (4 in cube and 2 in axial positions) and 4 × 2= 8 star points. The second-degree polynomial equation was used to determine the relationship between the independent variables and the response.

RESULTS AND DISCUSSION

Identification of significant factors using pbd

The influence of eight variables, in the production of l-glutaminase by the strain *b. Subtilis* jk-79 was investigated in 12 runs using pbd. Table 2 represents the PBD for the selected eight variables

Table 3. ANOVA for PBD.

Source	DF	Adj SS	Adj MS	F-Value	p-Value
Model	8	304153	38019	13.20	0.029*
Linear	8	304153	38019	13.20	0.029*
pH	1	34658	34658	12.03	0.040*
Temperature	1	632	632	0.22	0.671
Agitation speed	1	2647	2647	0.92	0.409
Incubation time	1	1971	1971	0.68	0.469
Inoculum size	1	4738	4738	1.64	0.290
Carbon source	1	79743	79743	27.68	0.013*
Nitrogen source	1	72007	72007	25.00	0.015*
Glutamine	1	107757	107757	37.40	0.009*
Error	3	8643	2881	-	-
Total	11	312795	-	-	-

*Significant. R^2 = 97.24%, Adj. R^2=89.87%, Pred. R^2= 55.79%.

Table 4. Actual and coded values of the factors employed in CCD.

Factor	Range of levels					
	Code	-2	-1	0	1	2
pH	A	3	5	7	9	11
Carbon source (fructose % w/v)	B	1.50	1.75	2.0	2.25	2.50
Nitrogen source (yeast extract % w/v)	C	0.5	1.0	1.5	2.0	2.5
Glutamine (% w/v)	D	0.5	1.5	2.5	3.5	4.5

and the corresponding response for L-glutaminase production (U/ml). Variations were observed in the production of L-glutaminase ranging from 150 to 666.67 U/ml. On the basis of analysis of variance (ANOVA), the most effective factors with high significance were in the order glutamine (H), fructose (F), yeast extract (G) and pH (A) (Table 3). The first order polynomial equation was derived representing L-glutaminase production as a function of independent variables

$$Y = 353.9 + 94.8\,H + 77.5\,G + 81.5\,F - 53.7\,A$$

Where, Y is the response (L-glutaminase production U/ml); H, G, F and A are independent variables of glutamine, fructose, yeast extract and pH, respectively with their corresponding co-efficients.

Statistical analysis of PBD demonstrated that the model F value of 13.2 is significant and among the significant factors, glutamine showed a remarkable effect on the production of L-glutaminase by *B. subtilis* JK-79. In the present model, R^2 was 97.24%, and only 2.77% variability was not explained. Thus, the present R^2 value reflected reliability of the model for predicting L-glutaminase production. The value of the adjusted determination coefficient (Adj R^2 = 0.8987) confirmed the significance of the model as well.

Optimization using CCD

RSM using CCD was adopted to understand the interactive effects of these four significant variables. Table 4 illustrates the details of actual and coded values employed in the CCD. The experimental trials were performed based on the CCD (Table 5) and the results obtained were fitted to a second order polynomial equation to explain the dependence of L-glutaminase production with the independent variables.

$$Y = +683.08 - 13.88 \times A + 10.05 \times B - 32.83 \times C - 5.88 \times D + 3.05 \times AB - 8.86 \times AC + 27.78 \times AD + 12.41 \times BC - 20.47 \times BD + 11.88 \times CD - 159.62 \times A^2 - 27.55 \times B^2 - 49.30 \times C^2 - 100.93 \times D^2$$

Where, Y is the response of L-glutaminase production, A, B, C and D are the coded values of pH, fructose, yeast extract and glutamine, respectively.

The analysis of variance of the quadratic regression model (Table 6) suggested that the model was very significant which was evident from the Fisher's F-test (F_{model} = 588.38) and a low probability value (P_{model}<0.0001). The p value for "lack of fit" (0.0957) also indicated that the quadratic model adequately fitted the data. In this model, A, B, C, D, AC, AD, BC, BD, CD, A^2, B^2, C^2, and D^2 are significant model terms. R^2 was found

Table 5. Observed response of CCD using four independent variables and six centre points.

Std.	Run	Factor 1	Factor 2	Factor 3	Factor 4	Response	Protein concentration (mg/ml)
		A:pH	B:Carbon source (% w/v)	C:Nitrogen source (% w/v)	D: Glutamine (%)	L-glutaminase activity (U/ml)	
26	1	0	0	0	0	686.67	14.12
2	2	1	-1	-1	-1	335.58	7.91
18	3	2	0	0	0	22.23	0.97
11	4	-1	1	-1	1	305.17	6.92
22	5	0	0	2	0	433.38	9.36
3	6	-1	1	-1	-1	458.87	9.98
24	7	0	0	0	2	268.38	5.28
29	8	0	0	0	0	691.27	14.98
20	9	0	2	0	0	592.28	12.23
9	10	-1	-1	-1	1	366.67	8.16
7	11	-1	1	1	-1	389.87	8.89
27	12	0	0	0	0	683.38	13.86
30	13	0	0	0	0	686.33	13.54
12	14	1	1	-1	1	372.27	8.38
14	15	1	-1	1	1	303.47	6.02
17	16	-2	0	0	0	68.33	3.42
25	17	0	0	0	0	677.87	13.02
23	18	0	0	0	-2	290.29	5.82
13	19	-1	-1	1	1	317.77	7.18
16	20	1	1	1	1	322.27	7.89
1	21	-1	-1	-1	-1	422.27	9.36
5	22	-1	-1	1	-1	308.33	6.02
6	23	1	-1	1	-1	218.87	4.33
8	24	1	1	1	-1	298.87	5.81
15	25	-1	1	1	1	318.87	7.61
21	26	0	0	-2	0	538.33	11.38
4	27	1	1	-1	-1	383.33	8.71
10	28	1	-1	-1	1	412.27	8.89
19	29	0	-2	0	0	553.83	11.41
28	30	0	0	0	0	672.97	13.34

to be 0.9982 indicating that the model was reliable.

3D response surfaces were generated to understand the interaction between independent variables. Figure 1*i-vi* shows the response surfaces and contour plots generated for the variation in the yields of L-glutaminase as a function of concentrations of two variables with the other two variables at their central value. The coordinates of the central point within the highest contour levels in each of the figures correspond to the optimum concentrations of the respective components. Evaluation of response surface curves and contour plots indicate the range of optimum conditions within the experimental area covered or show the way to conduct further experiments to achieve better results.

From the Figure 1i, it was evident that when the concentration of glutamine and nitrogen source were held at their middle values, the pH showed a parabolic response at the different concentrations of fructose with the highest yield of L-glutaminase obtained in the range of pH 7. Very low and high pH values were not favorable for enzyme production. Variation in fructose concentration has also followed a parabolic curve and optimum yield was in the range of 2.0 to 2.25%.

Similarly, the response behavior was analyzed between pH and yeast extract with the other two factors (glutamine and fructose) kept at their middle values. The production of L-glutaminae was affected by pH and followed a parabolic curve. Extreme conditions of pH decreased the production of the enzyme and the optimum pH was 7. However, the concentration of yeast extract also affected the response and the maximum L-glutaminase production was obtained in the range of 1 to 1.5% (Figure 1ii).

The 3D response surface and contour plot between pH and glutamine is represented in Figure 1iii. From these

Table 6. ANOVA for the CCD quadratic model.

Source	Analysis of variance table [Partial sum of squares - Type III]					
	Sum of squares	df	Mean Square	F Value	p-value Prob > F	Significance
Model	9.366E+005	14	66900.18	588.38	< 0.0001	Significant
A-pH	4622.87	1	4622.87	40.66	< 0.0001	-
B-Carbon source	2423.86	1	2423.86	21.32	0.0003	-
C-Nitrogen source	25873.32	1	25873.32	227.55	< 0.0001	-
D-Glutamine	828.96	1	828.96	7.29	0.0165	-
AB	148.90	1	148.90	1.31	0.2704	-
AC	1257.23	1	1257.23	11.06	0.0046	-
AD	12348.21	1	12348.21	108.60	< 0.0001	-
BC	2464.87	1	2464.87	21.68	0.0003	-
BD	6703.11	1	6703.11	58.95	< 0.0001	-
CD	2258.86	1	2258.86	19.87	0.0005	-
A^2	6.973E+005	1	6.973E+005	6132.63	< 0.0001	-
B^2	20742.70	1	20742.70	182.43	< 0.0001	-
C^2	66664.59	1	66664.59	586.31	< 0.0001	-
D^2	2.794E+005	1	2.794E+005	2457.38	< 0.0001	-
Residual	1705.54	15	113.70	-	-	-
Lack of Fit	1485.57	10	148.56	3.38	0.0957	Not significant
Pure Error	219.97	5	43.99	-	-	-
Cor Total	9.383E+005	29	-	-	-	-
Std. Dev.			10.66			
Mean			413.3			
C.V. %			2.58			
PRESS			8874			
R-Squared			0.9982			
Adj R-Squared			0.9965			
Pred R-Squared			0.9905			
Adeq Precision			88.267			

plots it was evident that lowest yield of L-glutaminase was obtained in the extreme pH conditions, that is, highly acidic and highly alkaline pH. The L-glutaminase production increased considerably when the pH approached its middle values and the optimum was in the range of 7. The L-glutaminase production was also affected by glutamine concentration and followed a parabolic curve. The yield was minimum at the very low and high concentration of glutamine and the highest production was obtained in the range of 2.5 and 3%.

The fructose and yeast extract concentration affected the L-glutaminase production and the optimum enzyme production was obtained in the fructose and yeast extract concentration of 2 to 2.5% and 1 to 1.5%, respectively (Figure 1iv). In the response behaviour of different concentrations of fructose and yeast extract (Figure 1v), the response pattern was found to be parabolic and the optimum production of L-glutaminase was obtained at the middle values. Figure 1vi demonstrates that the production pattern of L-glutaminase was parabolic with respect to glutamine concentration. In the case of yeast extract, the highest yield of the L-glutaminase was attained at the mid concentration and further increase in yeast extract concentration did not significantly change the production of L-glutaminase.

The optimum values of the independent variables were predicted using point prediction tool of design of expert software (Figure 2). Maximum L-glutaminase production of 688.5 U/ml under flask condition was obtained at the predicted optimal values of pH 6.9, fructose 2.1% (w/v), yeast extract 1.25% (w/v), and glutamine 2.47% (w/v). The maximum experimental L-glutaminase production was 691.27 U/ml thus indicating a strong correlation between them.

The L-glutaminase production under submerged fermentation by marine *B. subtilis* JK-79 was determined in optimized and unoptimized base medium, that is, Sea Water Glutamine (SWG) medium (Kiruthika and Saraswathy, 2014) and a 3.48 fold increase in glutaminase production was obtained by phase-wise

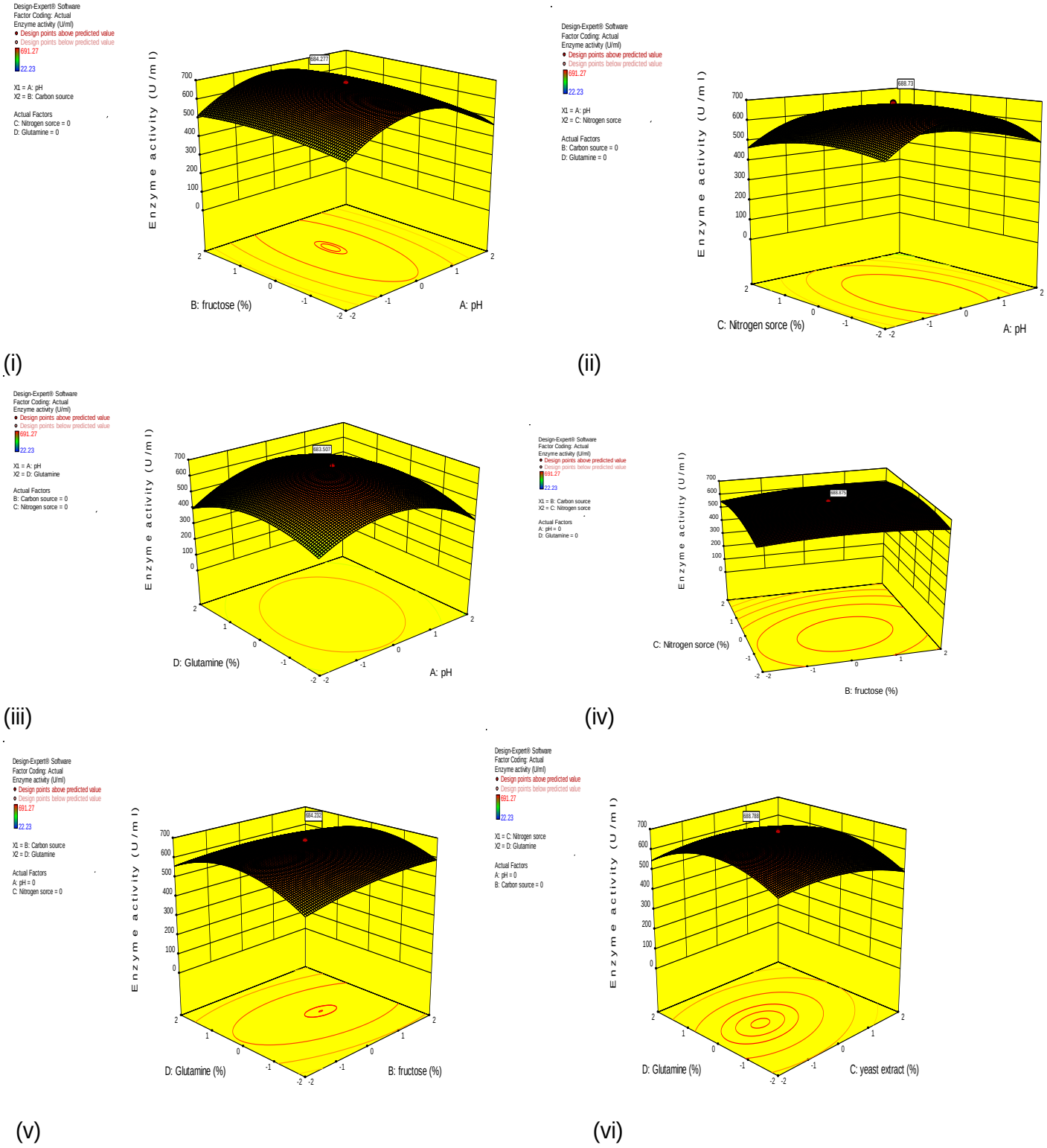

Figure 1. 3D response surface plots showing the interaction between the various components considered in the optimization. (i) Response behaviour of pH and fructose concentration under constant level of glutamine and nitrogen concentration. (ii) Response behaviour of pH and yeast extract concentration under constant level of glutamine and fructose concentration. (iii) Response behaviour of pH and glutamine concentration under constant level of yeast extract and fructose concentration. (iv) Response behaviour of fructose and yeast extract concentration under constant level of pH and glutamine concentration. (v) Response behaviour of fructose and glutamine concentration under constant level of pH and yeast extract concentration. (vi) Response behaviour of yeast extract and glutamine concentration under constant level of pH and fructose concentration.

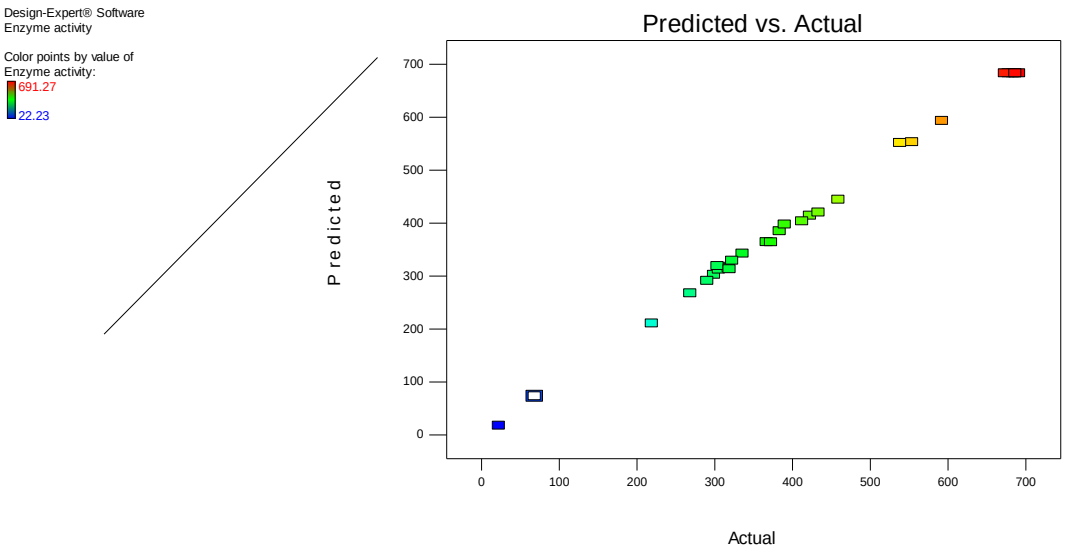

Figure 2. Plot between predicted and actual response of L-glutaminase production under submerged fermentation.

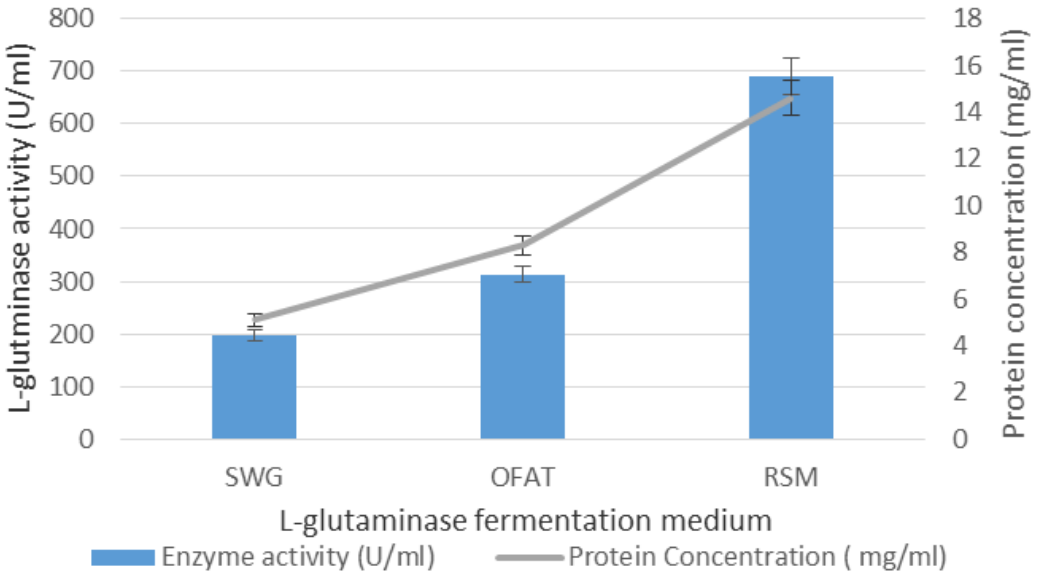

Figure 3. Comparision of L-glutaminase production by marine *Bacillus subtilis* JK-79 in basal and optimized medium.

optimizing the medium (Figure 3).

Several authors have reported an increased fold of L-glutaminase production by the application of RSM. Iyer and Singhal (2008) used a face centered central composite design (FCCCD) to enhance the L-glutaminase production and specific activity by 2.94 and 3.58 folds, respectively with *Z. rouxii*. Similarly, the authors have employed One Factor at a Time (OFAT) and FCCCD to design the optimized medium for *Providencia* spp. (Iyer and Singhal, 2009). Sathish and Prakasham (2010) found that a hybrid methodology adopted resulted in a significant improvement (47%) in the L-glutaminase yield by *B. subtilis* RSP-GLU.

Suresh et al. (2013) reported optimization of medium components through OFAT approach and FCCCD for the submerged production of L-glutaminase by *Serratia marcescens*. Pandian et al. (2014) reported the statistical optimization of medium composition for the production of glutaminase from *Alcaligenes faecalis* KLU102. RSM was used for optimization and the bacterium grown in the

optimized medium [arabinose (2%), skim milk (4%) and sodium chloride (2%)] yielded L-glutaminase activity of 1.34 IU/mg.

Jesuraj et al. (2017) has reported statistical optimization of L-glutaminase production by mutated strain *Aeromonas veronii* by PBD and CCD. The model was found to be a perfect fit in terms of maximizing enzyme yield, with the productivity improving at every stage to a fourfold output of enzyme (591.11 ±7.97 IU/mL) compared to the native strain (135±3.51 IU/mL).

Conclusion

Statistical optimization has enhanced L-glutaminase production under submerged fermentation by marine *B. subtilis* JK-79 (KC492745) (Kiruthika and Saraswathy, 2014) upto 3.48 fold when compared with the basal SWG medium. Thus, application of PBD and RSM for optimization studies proved to be an effective method for improving the L-glutaminase production. Results of this study revealed that marine *B. subtilis* JK-79 could be a promising source for L-glutaminase production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

American Cancer Society's Cancer Facts and Figures (2018). https://www.lls.org/http%3A/llsorg.prod.acquia-sites.com/facts-and-statistics/facts-and-statistics-overview/facts-and-statistics.

Chandrasekaran M (1996). Harnessing of marine microorganisms through soild state fermentation. Journal of Scientific and Industrial Research 55:468-471.

Douer D (2008). Is asparginase a critical component in the treatment of acute lymphoblastic leukemia? Best Practice and Research: Clinical Haemotology 21:647-658.

El-Ghonemy DH (2014). Microbial amidases and their industrial applications: A review. Journal of Medical Microbiology and Diagnosis 4(173):1-6.

Hensley CT, Wasti AT, De Berardinis RJ (2013). Glutamine and cancer: cell biology, physiology, and clinical opportunities. Journal of Clinical Investigation 123(9): 3678-84.

Imada A, Igarasi S, Nakahama K, Isono M (1973). Asparginase and glutaminase activities of microorganisms. Journal of General Microbiolgy 76:85-99.

Iyer P, Singhal RS (2008). Production of glutaminase (E.C.3.2.1.5) from *Zygosaccharomyces rouxii*: Statistical optimization using response surface methodology. Bioresource Techology 99:4300-4307.

Iyer P, Singhal RS (2009). Screening and selection of Marine isolate for L-glutaminase production and media optimization using response surface Methodology. Applied Biochemistry and Biotechnology 159:233-250.

Iyer P, Singhal RS (2010a). Glutaminase production using *Zygosaccharomyces rouxii* NRRRL-Y 2547: effect of aeration, agitation regimes and feeding strategies. Chemical Engineering and Technology 33:52-62.

Iyer P, Singhal RS (2010b). Isolation, screening, and selection of an L-glutaminase producer from soil and media optimization using a statistical approach. Biotechnology and Bioprocess Engineering 15:975-983.

Jesuraj SA, Sarker MM, Ming LC, Praya SM, Ravikumar M, Wui WT (2017). Enhancement of the production of $_{L}$-glutaminase, an anticancer enzyme, from *Aeromonas veronii* by adaptive and induced mutation techniques. PLOS ONE 12(8):e0181745.

Keerthi TR, Suresh PV, Sabu A, Kumar SR, Chandrasekaran, M (1999). Extracellular production of L-glutaminase by alkalophilic *Beauveria bassiana*-BTMF S10 isolated from marine sediment. World Journal of Microbiology and Biotechnology 15:751-752.

Kiruthika J, Saraswathy N (2014). Isolation and characterization of a novel L-glutaminase producing marine *Bacillus subtilis* JK-79. Asian Journal of Microbiology Biotechnology and Environmental Science 16(3):601-610.

Krishnakumar S, Alexis Rajan R, Ravikumar S (2011). Extracellular production of L-glutaminase by marine alkalophilic *Streptomyces sp.*-SBU1 isolated from Cape Comorin coast. Indian Journal of Geo-Marine Sciences 40:717-721.

Lowry OH, Rosebrough NN, Farr AL, Randall RY (1951). Protein measurement with the Folin Phenol reagent. Journal of Biological Chemistry 193:265-275.

Montgomery DC (2000). Design and Analysis of Experiments. Fifth Edition, John Wiley and Sons, New York, NY.

Myers RH, Montgomery DC, Anderson-Cook CM (2009). Response surface methodology: process and product optimization using designed experiments. Edition 3, John Wiley & Sons.

Noura El-Ahmady El-Naggar, Sara El-Ewasy M, Nancy El-Shweihy M (2014). Microbial L-asparginase as a potential therapeutic agent for the treatment of Acute Lymphoblastic Leukemia: The Pros and Cons. International Journal of Pharmacology 1:18.

Pandian SR, Deepak V, Sivasubramaniam SD, Nellaiah H, Sundar K (2014). Optimization and purification of anticancer enzyme L-glutaminase from *Alcaligenes faecalis* KLU102. Biologia 69:1644-1651.

Sabu A (2003). Sources, properties and applications of marine therapeutic enzymes. Indian Journal of Biotechnology 11:1211-1225.

Sathish T, Prakasam RS (2010). Enrichment of glutaminase production by *Bacillus subtilis* RSP-GLU in submerged cultivation based on neural network-genetic algorithm approach. Journal of Chemical Technology and Biotechnology 85:50-58.

Sathish T, Prakasam RS (2013). Influence of fermentation process parameters on L-glutaminase production by *Bacillus subtilis* RSP-GLU. International Journal of Pharmaceutical and Chemical Science 1:625-631.

Sivakumar K, Sahu MK, Manivel PR, Kannan L (2006). Optimum conditions for L-glutaminase production by actinomycete strain isolated from estuarine fish, *Chanoschanos* (Forskal, 1775) Indian Journal of Experimental Biology 44:256-258.

Souba WW (1993). Glutamine and cancer. Annals of Surgery 218:715-728.

Suresh Kumar S, Muthuvelayudham R, Viruthagiri T (2013). Production and optimization of L-glutaminase (EC.3.5.1.2) by *Serratia marcescens* using wheat bran under statistical designs. Journal of Chemical, Biological and Physical Sciences 3:2601-2612.

Unissa R, Sudhakar M, Sunil Kumar Reddy A, Naga Sravanthi K (2014). A review on biochemical and therapeutic aspects of glutaminase. International Journal of Pharmaceutucal Science and Research 5:4617-34.

Vellard M (2003). The enzyme as drug: application of enzymes as pharmaceuticals. Current Opinion in Biotechnology 14:444-450.

Vijayan N, Swapna TS, Haridas M Sabu A (2017). 11-Therapeutic enzymes – L-glutaminase. Current developments in Biotechnology and Bioengineering pp. 233-248.

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Seroprevalence of contagious bovine pleuropneumonia and contagious caprine pleuropneumonia in the Middle-Belt of Nigeria

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The aim of the study was to establish the seroprevalence of contagious bovine pleuropneumonia (CBPP) and contagious caprine pleuropneumonia (CCPP) among cattle and small ruminants received at abattoirs in Ilorin, Nigeria. A total of 643 blood samples were taken from 324 cattle and 319 sheep and goats. These samples were screened for CBPP and CCPP using rapid latex agglutination tests. The majority were from the north of Nigeria. Sera from cattle were screened for the causative mycoplasma of CBPP, *Mycoplasma mycoides* subsp. *mycoide*s (Mmm) and small ruminants were screened for the presence of *Mycoplasma capricolum* subsp. c*apripneumoniae* (Mccp), the cause of CCPP, using specific latex agglutination tests. Clinical signs including specific clinical signs associated with CBPP were also assessed. A prevalence rate of just over 56% was recorded for CBPP. In all, 22 of 324 cattle showed clinical signs but only 10 showed specific clinical signs associated with CBPP infection such as dyspnoea, nasal discharge and cough. A CCPP prevalence rate of about 33% was found among the small ruminants. In total, 52 of 319 small ruminants showed clinical signs of disease but only 22 of these were associated with seropositivity. The high seroprevalence of CBPP and CCPP in this study is suggestive of extensive CBPP and CCPP infection in Ilorin, Nigeria.

Key words: Abattoir survey, Nigeria, contagious bovine pleuropneumonia (CBPP), contagious caprine pleuropneumonia (CCPP), seroprevalence.

INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is a highly contagious disease of cattle associated with consolidation and 'marbling' of the lung, fibrinous pleurisy and accumulation of pleural fluid (OIE, 2013). Transmission

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of the disease is through intimate contact between infected and susceptible cattle due to the inhalation of infected droplets released during coughing, or in nasal discharges from infected animals (OIE, 2013). Nigeria's status was regarded as CBPP-free in 1965 which had been achieved by a 10-year policy of harmonized disease reporting, vaccination, prompt laboratory diagnosis, quarantine and slaughter (Griffin and Laing, 1966). However, since that time, CBPP cases have re-emerged with the prevalence increasing across different regions of Nigeria (March et al., 2003; Foluso, 2003; Mailafia et al., 2010). In the humid zones, higher herd prevalence has been observed than in arid zones due to differences in the rate of contact between cattle because of their respective production systems (Masiga et al., 1996; Mariner et al., 2005).

Contagious caprine pleuropneumonia (CCPP) is a highly contagious disease of goats and sheep and was originally reported in Algeria in 1873. Its devastating nature and cause of economic losses in the livestock industry makes it a globally notifiable disease (Manso-Silvan et al., 2009). CCPP is pandemic in parts of Asia, Middle East and Africa and it is one of the major threats to the small ruminant industries of developing countries (OIE, 2013). Only 20 countries previously reported the isolation of the causative mycoplasma possibly because of the scarcity of laboratory expertise. However, its isolation has now been confirmed in many regions such as China, Mauritius, Tajikistan, Nigeria, and India using improved detection methods such as the agglutination test, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) (Nicholas and Churchward, 2012).

There are only a few recent reports describing the prevalence of CCPP in Nigeria compared to CBPP (Egwu et al., 2012; Akwuobu et al., 2014) and none in the southern part of the country. Following the eradication of rinderpest from cattle in 2011, there is now significant global activity in controlling peste des petits ruminants (OIE, 2013). Since CCPP and this disease can show similar clinical signs it will become increasingly important to differentiate between the two.

Available veterinary records show that CBPP is endemic in Nigeria and that it spreads via movement of trade cattle, seasonal migration and transhuman activities (Aliyu et al., 2000; Foluso, 2003; Ajuwape et al., 2004; Olorunshola et al., 2017). Also outbreaks of CBPP still occur particularly in the northern region which harbours three quarters of the country's 16.3 million cattle (PACE, 2004). CBPP is caused by the *Mycoplasma mycoides* subsp. *mycoide*s bacteria which belong to a cluster consisting of five closely related mycoplasmas, namely, *Mycoplasma mycoides* subsp. *capri* (Mmc), *Mycoplasma capricolum* subsp. c*apripneumoniae* (Mccp), *Mycoplasma capricolum* subsp. *capricolum* (Mcc) and *Mycoplasma leachii* (Ml) (Thiaucourt et al., 2011).

Nigeria has a population of between 8 and 13 million sheep with most being found in the northern region and about 3.4 million located in the humid (southern) region. The goat population is about 22-26 million with 6.6 million reported to be in southern region while 20 million are in northern region (Lawal-Adebowale and Alarima, 2011). Breeds of sheep are mainly the indigenous West African Dwarf (WAD) sheep, Due, Balami and Yanks. Among the listed indigenous breeds, Uda, Balami and Yankasa are mostly widespread in the northern region while the WAD breed is common in the southern region. The breeds of goat in Nigeria are also mostly indigenous, and these include the WAD, Sahel/desert known as West African Long-Legged goat, and Sokoto Red/Maradi (Lawal-Adebowale and Alarima, 2011). The Sahel or desert goat and Sokoto Red are the most commont in the north while the WAD is more common in Southern Nigeria (FAO, 2004).

There have been several studies on the epidemiology of CBPP in Nigeria compared with CCPP (Aliyu et al., 2003; Ajuwape et al., 2004; Babalobi, 2011); however, scant information about CCPP is available in the mid-belt region of the country. This study was essentially carried out in Ilorin which is North Central Nigeria. While most of the animals used are from North West Nigeria, effects of relocation especially migration from North West Nigeria to North Central Nigeria make this study very relevant. Furthermore, information on the prevalence of CCPP in Nigeria is still sparse (Ajuwape et al., 2004; Babalobi, 2011). The aim of this study was to provide an up to date epidemiological survey based on abattoir data, regarding the seroprevalence of CBPP and CCPP in the Ilorin region of Nigeria.

MATERIALS AND METHODS

Study area

Ilorin is the capital of Kwara State which lies on the plain in the southwest of Nigeria at Latitude 8° 30' and 8° 50' N and Longitude 4° 20' and 4°35' E. The city occupies an area of about 468 km^2 situated within the forest and the guinea savannah regions of Nigeria. The Ilorin climate is tropical, being influenced by the two prevailing trade winds and experiences an annual rainfall of between 1000 and 1500 mm with daily average temperatures of 25, 27 and 22.5°C in January, May and September, respectively. The sampling location for this study was the central abattoir at Ipata, drawing animals from a pool of cattle markets in the local communities of Sango, Oke-ose, Oko-olowo, Alapa, Igbeti, Sadu, Sare, Ajase and Ipata.

Animals, sample collection and study design

A total of 643 blood samples from ruminants, 324 from cattle and 319 from small ruminants (44 sheep and 275 goats) were collected from the Ipata central abattoir. Collected samples of about 3 to 5 ml were kept in labelled sterile bottles and transported to the laboratory in cold boxes containing ice packs and later centrifuged for serum separation before serological testing. The blood sample collections took place from 14 to 24th of December 2016. Relevant information concerning the animals including species, age, sex,

Table 1. Sero- prevalence of CBPP in Nigerian cattle samples.

Parameter		Total No. of cattle	No. positive (%)	No. negative (%)
Sex of cattle	Males	4 (1.2)	1 (0.3)	3 (0.9)
	Females	320 (98.8)	181 (55.9)	139 (42.9)
	Total	324 (100)	182 (56.2)	142 (43.8)
Region of origin	North	226 (69.8)	125 (38.6)	101 (31.2)
	South	95 (29.3)	55 (17)	40 (12.3)
	Border area	3 (0.9)	2 (0.6)	1 (0.3)
	Total	324 (100)	182 (56.2)	142 (43.8)
Breed of cattle	Red Sokoto	50 (15.4)	31 (9.6)	19 (5.9)
	Sokoto gudali	110 (34.0)	63 (19.4)	47 (14.5)
	White fulani	98 (30.2)	55 (17.0)	43 (13.3)
	Red Bororo	62 (19.1)	31 (9.6)	31 (9.6)
	Other	4 (1.2)	2 (0.6)	2 (0.6)
	Total	324 (100)	182 (56.2)	142 (43.8)
Observations of clinical signs	[1]Any signs observed	22 (6.8)	[a]6 (1.9)	[b]16 (4.9)
	[1]No signs observed	302 (93.2)	176 (54.3)	126 (38.9)
	Total (%)	324 (100.0)	182 (56.2)	142 (43.8)
Observations of specific respiratory signs	[2]Respiratory signs observed	10 (3.1)	3 (0.9)	7 (2.2)
	[2]Respiratory signs not observed	314 (96.9)	179 (55.2)	135 (41.7)
	Total (%)	324 (100.0)	182 (56.2)	142 (43.8)

[1]All clinical signs including respiratory signs, diarrhoea, lameness, tick infestation and other skin lesions. [2]Clinical signs of respiratory disease including nasal discharge, respiratory dyspnoea, cough, but also lung lesions *post mortem.* [ab]Values with different alphabetical superscripts were significantly different (P<0.05).

breed, source, weight, physical appearance and health status measured using classic respiratory signs such as dyspnoea, cough, nasal discharge and emaciation were collected using a structured questionnaire. Cattle carcases were examined for the presence of lung lesions at *post mortem.*

Serological tests

Rapid latex agglutination tests were used for the detection of both CBPP and CCPP antibodies. These tests had previously proved effective for screening for these diseases in Afghanistan (Bahir et al., 2017). For CBPP detection in cattle serum, the BoviLAT (PA6223) and for CCPP the CapriLAT (RAI6224) tests were used. Both test kits were kindly supplied by the Mycoplasma group at the Laboratory Agency (Weybridge), Addlestone, Surrey, UK. The procedures were carried out according to the manufacturer's instructions. The test slides were read for the presence of agglutination after between 1 and a maximum of 3 min. Each animal was observed for clinical signs on presentation at the abattoir which were recorded along with the presenting clinical signs for each animal. The latter included fever (40-42°C), cough and dyspnoea. Positive and negative control sera provided with the kits were tested every 24 samples to monitor test variance and the tests performed in line with previously published data (Nicholas et al., 1996; Ayling et al., 1999a, b; Bahir et al., 2017). Data were analysed by Chi square using SPSS (v.20)

RESULTS

Of the samples that were positive for agglutination after 3 min, 16.5 and 42.7% were positive after 1 to 2 min, respectively.

Cattle

The sero-prevalence of CBPP amongst the sampled cattle was 56.2% (182 of 342). Table 1 shows the cattle sero-prevalence data disaggregated by test result, sex, breed and geographical origin. 320 of the cattle sampled were female while only four were male. Although the prevalence of CBPP was numerically higher in cattle originating from the northern region (38.6%) compared to the south (17.0%) this was not statistically significant ($p \geq 0.05$). 320 of the 324 cattle were from the 4 breeds Red Sokoto (50), Sokoto gudali (110), White Fulani (98) and Red Buroro (62), with no significant differences in prevalence between breeds (Table 1) ($p \geq 0.05$). The age range recorded for the sampled cattle was between 1 and 7 years; 310 were within the age range of 1 to 3 years while 14 were within the 4 to 7 years age range. The live

Table 2. Sero-prevalence of CCPP in Nigerian small ruminants.

Parameter		Total No. of animals	No. positive (%)	No. negative (%)
Species	Goat	275 (86.2)	90 (28.2)	185 (58)
	Sheep	44 (13.8)	15 (4.7)	29 (9.1)
	Total	319 (100)	105 (32.9)	214 (67.1)
Sex	Male	266 (83.4)	82 (25.7)	184 (57.7)
	Female	53 (16.6)	23 (7.2)	30 (9.4)
	Total	319 (100)	105 (32.9)	214 (67.1)
Region of origin	North	180 (56.4)	55 (17.2)	125 (39.2)
	South	139 (43.6)	50 (15.7)	89 (27.9)
	Total	319 (100)	105 (32.9)	214 (67.1)
Breed	Red Sokoto	178 (55.8)	58 (18.2)	120 (37.6)
	West African Dwarf	93 (29.2)	28 (8.8)	65 (20.4)
	Belami	41 (12.8)	18 (5.6)	23 (7.2)
	Other	7 (2.2)	1 (0.3)	6 (1.9)
	Total	319 (100)	105 (32.9)	214 (67.1)
Observations of clinical signs	Signs observed	52 (16.3)	22 (6.9)	30 (9.4)
	No signs observed	267 (83.7)	83 (26.0)	184 (57.7)
	Total	319 (100)	105 (32.9)	214 (67.1)

weights of the cattle varied between 100 and 200 kg. The clinical observation data are also shown in Table 1. In all, 22 of the 324 cattle were recorded as showing clinical signs, 10 of which showed specific respiratory signs associated with possible CBPP infection, that is, dyspnoea, nasal discharge and cough with or without lung lesions seen subsequently at *post mortem*.

Small ruminants (SR)

The SR seroprevalence data are shown in Table 2. Of the 319 SR, 275 (86.2%) were caprine while 44 (13.8%) were ovine. In total, 32.9% (105) tested positive while 67.1% (214) were negative (Table 2). Their respective ages ranged from 5 to 35 months while their weights ranged from 5 to 45 kg. Of the 319 SR, 126 (39.5%) were brown in colour, 92 (28.8%) were black, 67 (21.0%) were white while 34 (10.7%) contained mixed colours. Most SR originated from Northern Nigeria (56.4%). There were no significant differences ($p \geq 0.05$) in the proportion of seropositives between the breeds (Table 2). Of the 319 SR, 52 (16.3%) showed signs of unspecified clinical disease while 267 (83.7%) did not.

DISCUSSION

Since it was first reported in 1924, CBPP has been endemic in Nigeria (Foluso, 2003). Reasons have been associated with transhumance, nomadism, and other inadequate control measures (Egwu et al., 1996). For instance, Billy et al. (2017) reported an overall sero-prevalence of 26.0%. The herd level prevalence of 54.7% with 30.2% seropositivity to CBPP within agro-pastoral areas was documented by Suleiman et al. (2015). Danbirni et al. (2010) reported 47% sero-prevalence in a herd of cattle with combined infection with TB. Okaiyeto et al. (2011) reported a sero-prevalence of 16.7 and 17.5% for adults and young cattle, respectively, in a herd of cattle with CBPP outbreak. Musa et al. (2016) also reported 3.33% prevalence of CBPP from lung samples from abattoirs in North Eastern Nigeria. The result of this study also differ from the work of Nawathe (1992) and Adamu and Aliyu (2006) who in their separate studies recorded a lower sero-prevalence of 0.52 and 0.33%, respectively in Borno State and Aliyu et al. (2000) who recorded a sero-prevalence of 0.29% in 5 other states in northern part of Nigeria.

In this survey, the overall seroprevalence of CBPP was 182 (56.2) in cattle, CCPP was 90 (28.2) in goats, 15 (4.7) in sheep, and overall seroprevalence of 105 (32.9) in small ruminants, this make our reported prevalence not only higher than others, but our survey also captured the small ruminants. The higher seroprevalence rate recorded in this study could be as a result of the inadequate prevention and control measures that resulted in absence or irregular vaccination programmes for cattle over the

years, as well as the introduction of infected cattle into the areas (particularly through transhumance and nomadism) that were initially thought to be free of the disease (Aliyu et al., 2000). It could also be as a result of epidemiological trend of the disease with the presence of carriers in some herds which might not have been detected clinically and hence the maintenance and gradual spread of the disease (Egwu et al., 1996).

Reliable diagnosis of these two diseases by serology is complex, as the performance of available tests varies with the stage of infection and/or disease (Muuka et al., 2011). Thus the fact that the presence of antibodies did not coincide well with the detection of clinical signs is not surprising. A complement fixation test (CFT) and competitive ELISA test for CBPP are approved by OIE (2018a, b) for screening purposes but are generally not considered sufficiently reliable for diagnosis in individual animals because of variable sensitivities and/or specificities.

The CFT is reported as being highly specific at 98% (Bellini et al., 1998) but much lower sensitivity at between 55 and 60%. According to March et al. (2003), CBPP-CFT-false negative results may occur but LAT is cheaper, highly specific and easy to apply in the field, without any specialist training or equipment. The LAT used here specifically targets antibodies to a capsular polysaccharide (CPS) and is reported to have equivalent specificity to the CFT, that is, in the high 90%s, but with lower sensitivity (Ayling et al., 1999a, b). Therefore, in interpreting the present data, an apparent seroprevalence of 56.2% is likely to have under-estimated the true prevalence of *M. mycoides* subsp. *mycoide*s CPS antibodies. Nevertheless, a value of 56.2% in cattle and 32.9% in small ruminants reported in this study represents a very significant prevalence which suggests that *M. mycoides* subsp. *mycoide*s infection is very common in these regions of Nigeria.

In the case of CCPP, the LAT used was as described by March et al. (2002) which is based on recognition of the *M. capricolum* subsp. c*apripneumoniae* CPS antigen and is a recognised test by OIE (2018b). This test has a high specificity (March et al., 2002) of approximately 99% (APHA, Weybridge, UK, personal communication). A close agreement of 96% was found between the LAT and the CFT in 54 sera from goats experimentally infected with *M. capricolum* subsp. c*apripneumoniae* (Abdel-Hamid et al., 2016). The test is considered to be highly sensitive particularly in the early stages of infection (March et al., 2002; OIE 2018a, b).

In general, despite some limitations in sensitivity, the specific LATs for *M. mycoides* subsp. *mycoide*s and *M. capricolum* subsp. c*apripneumoniae* are regarded as useful tests for field screening and have the advantage that they can be used relatively easily in the field with little need for specialist training or equipment. The speed of agglutination reactions was consistent with those previously published (Bahir et al., 2017).

CBPP in cattle

The seroprevalence rate of 56.2% reported here, is generally higher than previous abattoir seroprevalence studies of CBPP reported in Nigeria and other parts of Africa of between 10 and 32% (Griffin and Laing, 1966, Aliyu et al., 2003; Okaiyeto et al., 2011). This suggests an increasing trend over time, which is in agreement with the annual report by OIE (2013) which stated that CBPP is gradually spreading across Nigerian states from the northern region to other regions because of constant migration of cattle herders. It is known that there is significant migration of cattle herders between regions and in particular, there is evidence that the disease is increasingly moving from the north towards the south (Foluso, 2003; Mailafia et al., 2010). In the present study, the majority of the cattle were from the north and the seroprevalence was numerically higher in these cattle (38.6%) compared to the southern (17.0%) but this was not statistically significant.

In this study, the majority of the cattle were female with just 1.2% male. This is probably because females are normally kept in higher numbers for rearing than males. Because of the small numbers of males any gender differential in seroprevalence could not be determined. Egwu et al. (2012) reported a lack of gender predisposition to CBPP infection.

Just 22 of the 324 cattle showed clinical signs of disease but only 10 of these included respiratory signs indicative of possible CBPP, which included dyspnoea, nasal discharge and cough with or without emaciation. More than half (54.3%) of the animals certified as clinically healthy when inspected pre-slaughter, were sero-positive to *M. mycoides* subsp. *mycoide*s. This could be attributed to late disease manifestation, high immunity levels of the animal or as a result of being an asymptomatic carrier or latent carrier; the latter state usually occurring when an affected animal partially recovers after 3 to 4 weeks (OIE, 2011).

CCPP in small ruminants

The overall serological prevalence of *M. capricolum* subsp. c*apripneumoniae* in small ruminants was 32.9% and this suggests that there is significant circulation of *M. capricolum* subsp. c*apripneumoniae* in Nigeria. These results are consistent with previous reports of CCPP prevalence in the north of Nigeria (Egwu et al., 2012; Chinedu et al., 2014). Also, significant prevalence rates of CCPP have been reported in other parts of Africa. For example, Hadush et al. (2009) reported a CCPP prevalence rate of 32.68% and Mekuria et al. (2008), reported a CCPP prevalence rate of 18.62%, both in Ethiopia.

In the present study there was a higher prevalence of CCPP in goats (28.2%) compared to sheep (4.7%). This

is to be expected because *M. capricolum* subsp. c*apripneumoniae* has most often been associated with goats whereas infection in sheep is often associated with their close proximity to goats, goats being regarded as more susceptible than sheep to mycoplasmas (Akwuobu et al., 2014). CCPP seroprevalence tended to be higher in males than females (25.7% vs. 7.2%) although this was not statistically significant. This effect has been previously reported by Akwuobu et al. (2014) who suggested that males may be more susceptible than females. This is totally at variance to the submission of Torsson et al. (2017) who argued that females are more susceptible to these diseases due to the length of time they are kept for the purpose of reproduction.

More (56.4%) of the SR originated from the north than from the south (43.6%), a similar finding to the studies of Lawal-Adebowale and Alarima (2011) and Akwuobu et al. (2014). The geography of the northern region is more conducive to SR production due to the larger areas of suitable agroclimatic conditions in terms of adequate rainfall, a longer dry season and lighter sandy soil. Highest seropositivity was noted amongst the Red Sokoto which was also the largest population, followed by the WAD and Balami. 52 of the total 319 SR (16.3%) showed evidence of clinical signs of disease but only 22 of these were sero-positive for CCPP. Thus the majority of sero-positives did not show signs of clinical disease again suggesting a carrier state as in cattle.

In conclusion, the present study demonstrated the presence of both *M. mycoides* subsp. *mycoide*s and *M. capricolum* subsp. c*apripneumoniae* in animals originating from both the north and south of the country. This suggests that the two mycoplasma species are well established in both cattle and small ruminants in Nigeria. In the past, efforts to control these diseases have not been effective due both to lack of sustained and effective control strategies, doubts about vaccine safety and efficacy and, an antipathy to the use of antibiotics for CBPP in cattle. An assessment of the economic impact of these diseases would be appropriate to determine the cost effectiveness of control programmes to rid Nigeria of these potentially devastating diseases.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Abdel-Hamid N, Abdel-Mortada O, Abd Elhady H, Farouk R (2016). Bacteriological examination and diagnostic performance of some serological tests used in diagnosis of small ruminant brucellosis. Veterinary Medical Journal Giza 62(4):11-21.

Ajuwape ATP, Adetosoye AI, Ikheloa JO, Alaka OO, Taiwo O, Talabi OA, Otesile EB, Ojo MO (2004). Pathogenicity of Mycoplasma capricolum subsp. capripneumoniae for cattle immunesuppressed by Trypanasoma congolence. Tropical Veterinarian 22(1):29-36.

Akwuobu CA, Ayling RD, Chah KF, Oboegbulem SI (2014). Studies into the prevalence of Mycoplasma species in small ruminants in Benue State, North-central Nigeria. Springer: Tropical Animal Health and Production 46(6):1087-1092.

Aliyu MM, Obi, TU, Egwu GO (2000). Prevalence of Contagious Bovine Pleuropneumonia (CBPP) in Northern Nigeria. Preventive Veterinary Medicine 47:263-269.

Aliyu MM, Obi TU, Oladosu LA, Egwu GO, Ameh JA (2003). The use of competitive enzyme linked immuno-sorbent assay in combination with abattoir survey for CBPP surveillance in Nigeria. Tropical Animal Health and Production 21:35-41.

Ayling RD, Regalla J, Nicholas RAJ (1999a). A field test for detecting antibodies to Mycoplasma mycoides subsp.small colony type using the latex slide agglutination test. In: COST 826 Agriculture and biotechnology. Mycoplasmas of ruminants: pathogenicity, diagnostics, epidemiology and molecular genetics Vol III Stipkovits, L., Rosengarten, R., and Frey,J., eds. Luxembourg; Office for official publications of the European Communities 3:155-158.

Ayling RD, Miles RJ, Regalla J, Nicholas RAJ (1999b). Development of improved serological tests for the diagnosis of CBPP. Proceedings of the International Symposium on Mycoplasma of Ruminants. Toulouse, France June, 2nd-4th, P 35. https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_scah_out63_en.pdf

Babalobi OO (2011). Participatory Epizootiology Research of Settled Pastoralists in Igangan Grazing Reserve, Southern Guinea Agro-Pastoral Zone, Oyo State, Nigeria: First Report Nigerian Veterinary Journal 32(1):16-20.

Bahir W, Omar O, Rosales RS, Hlusek M, Ziay G, Schauwers W, Whatmore AM, Nicholas RAJ (2017). Search for OIE-listed ruminant mycoplasma diseases in Afghanistan. BMC Veterinary Research 13(1):149.

Bellini S, Giovannini A, Di Francesco C, Tittarelli M, Caporale V (1998). Sensitivity and specificity of serological and bacteriological tests for contagious bovine pleuropneumonia. Revision Science Technology 17:654-670.

Billy IL, Balami AG, Sackey AKB, Tekdek LB, Sa'idu SNA, Okaiyeto SO (2017). Sero-Prevalence of Contagious Bovine Pleuropneumonia in Three Senatorial District of Kaduna State, Nigeria Using Latex Agglutination Test. World Veterinary Journal 7(2):65-73.

Chinedu AA, Roger DA, Kennedy FC, Stephen IO (2014). Studies into the prevalence of Mycoplasma species in small ruminants in Benue State, North-central Nigeria. Springer: Tropical Animal Health Production 46:1087-1092

Danbirni S, Okaiyeto SO, Pewan SB, Kudi AC (2010). Concurrent infection of Contagious Bovine Pleuropneumonia and Bovine tuberculosis in Bunaji nomadic cows. Research Journal of Animal Science 4(1):23-25.

Egwu GO, Nicholas RAJ, Ameh JA, Bashiruddin JB (1996). Contagious bovine pleuropneumonia. An update. Veterinary Bulletin 66:875-888.

Egwu GO, Adamu M, Mshelia GD, Bukar-Kolo YM (2012). A sustainable laboratory approach for contagious bovine pleuropneumonia (CBPP) monitoring in Nigeria: comparison between two serological tests in an endemic area complimented with post mortem lesions. African Journal of Microbiology Research 6(30):5890-5895.

Foluso EF (2003). Status of contagious bovine pleuropneumonia (CBPP) in Nigeria with emphasis on control strategies. Proceedings of the FAO-OIE-AU/IBAR-IAEA Consultative Group on CBPP 3rd Meeting, Towards Sustainable CBPP Control Programmes in Africa, Nov. 12-14, 2003, Rome P 29. http://www.fao.org/docrep/007/y5510e/y5510e0u.htm

Food and Agricultural Organization (FAO) (2004). Statistical Yearbook of the Food and Agricultural Organization for the United Nations FAO. 2004. The State of Food and Agriculture Source: FAO, Statistics Division http://faostat.fao.org.

Griffin RM, Laing DF (1966). Contagious bovine pleuropneumonia in Northern Nigeria. Bulletin of Epizootic Disease in Africa 14:255-279.

Hadush B, Eshetu L, Mengistu W, Hailesilassie M (2009). Seroprevalence of contagious caprine pleuropneumonia in Kefta Humera, Alamata (Tigray) and Aba-'ala (Afar), Northern Ethiopia. Tropical Animal Health and Production 41:803-806.

Lawal-Adebowale OA, Alarima CI (2011). Challenges of small ruminants' production in selected urban communities of Abeokuta,

Ogun State. Agriculturale Conspectus Scientificus 76(2):129-134. Published by Faculty of Agriculture, University of Zagreb, Croatia. Available online at http://hrcak.srce.hr/index.php?show=clanak&id_clanak_jezik=105190.

Manso-Silván L, Vilei EM, Sachse K, Djordjevic SP, Thiaucourt F, Frey J (2009). Proposal to assign Mycoplasma leachii sp. nov. As a new species designation for Mycoplasma sp. bovine group 7 of leach, and reclassification of Mycoplasma mycoides subsp. mycoides LC as a serovar of Mycoplasma mycoides subsp. Capri. International Journal of Systematic and Evolutionary Microbiology 59(6):1353-1358.

March JB, Harrison JC, Borich SM (2002). Humoral responses following experimental infection of goats with *Mycoplasma capricolum* subsp. *capripneumoniae*. Veterinary Microbiology 84:29-45.

March JB, Kerr K, Lema B (2003). Rapid detection of contagious bovine pleuropneumonia by a Mycoplasma mycoides subsp. mycoides small colony capsular polysaccharide-specific antigen detection latex agglutination test. Clinical Diagnostic Laboratory Immunology 10:233-340.

Mailafia S, Onakpa MM, Dandam KP (2010). A ten year study on the prevalence of ruminant diseases encountered at the Ministry of the Federal Capital Territory veterinary clinics Gwagwalada, Abuja, Nigeria. Sahel Journal of Veterinary Science 9(2):4-6.

Mariner JC, McDermott J, Heesterbeek JAP, Thomson G and Martin SW (2005). A model of contagious bovine pleuropneumonia transmission dynamics in East Africa. Preventive Veterinary Medicine 73(1):55-74.

Masiga WN, Domenech J, Windsor RS (1996). Manifestation and epidemiology of contagious bovine pleuropneumonia in Africa. Revue Scientifique et Technique 15(4):1283-1308.

Mekuria S, Zerihun A, Gebre-Egziabher B, Tibbo M (2008). Participatory investigation of Contagious Caprine Pleuropneumonia (CCPP) in goats in the Hammer and Benna-Tsemay districts of southern Ethiopia. Tropical Animal Health and Production 40:571-82.

Muuka G, Hang'ombe BN, Nalubamba KS, Kabilika S, Mwambazi L, Muma JB (2011). Comparison of complement fixation test, competitive ELISA and LppQ ELISA with post-mortem findings in the diagnosis of contagious bovine pleuropneumonia (CBPP). Tropical Animal Health and Production 43:1057-1062.

Nawathe DR (1992). Resurgence of Contagious Bovine Pleuropneumonia in Nigeria. Revue Science Technical Office International Epizootics 11:799-804.

Nicholas R, Churchward C (2012). Contagious caprine pleuropneumonia: new aspects of an old disease Transboudary Emerging Disease 59(3):189-196.

Nicholas RAJ, Santini FG, Clark KM, Palmer NM, DeSantis P, Bashiruddin JB (1996). A comparison of serological tests and gross lung pathology for detecting contagious bovine pleuropneumonia in two groups of Italian cattle. Veterinary Record 139:89-93.

Office International Des Epizooties (OIE) (2011). General disease information sheet: Contagious bovine pleuropneumonia. https://www.oie.int/doc/ged/D13980.PDF

Office International Des Epizooties (OIE) (2018a). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals Chapter 3.4.8 pp. 1097-2018.

Office International Des Epizooties (OIE) (2018b). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals Chapter 3.7.4. pp. 1441-1455.

Okaiyeto SO, Danbirni S, Allam L, Akam E, Pewan SB, Kudi AC (2011). On-farm diagnosis of contagious bovine pleuropneumonia in nomadic herds using latex agglutination test. Journal Veterinary Medicine and Animal Health 3(5):62-66.

Olorunshola ID, Andrew RP, Scacchia M, Nicholas RAJ (2017). Contagious bovine pleuropneumonia – never out of Africa? CAB Reviews 12(019):1-7.

Pan African Control of Epizootics (PACE) (2004). Newsletter No. 6:2. www.au-ibar.org

Suleiman A, Bello M, Dzikwi AA, Talba AM, Grema HA, Geidam YA (2015). Serological prevalence of contagious bovine pleuropneumonia in agro-pastoral areas of Nigeria. Tropical Animal Health and Production 47(6):1033-1042.

Thiaucourt F, Manso-Silvan L, Salah W, Barbe V, Vacherie B, Jacob D, Breton M, Dupuy V, Lomenech AM, Blanchard A, Sirand-Pugnet P (2011). Mycoplasma mycoides, from "mycoides small colony" to"capri". A micro evolutionary Perspective. BioMed Central Genomics 12:114.

Torsson E, Berg M, Misinzo G, Herbe I, Kgotlele T, Päärni M, Roos N, Blomström A, Ståhl K, Wensman JJ (2017). Seroprevalence and risk factors for peste des petits ruminants and selected differential diagnosis in sheep and goats in Tanzania. Infection Ecology and Epidemiology 7:1368336

Office International Des Epizooties (OIE) (2013). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE, Paris. https://www.oie.int/doc/ged/D12009.PDF (Accessed 16th January 2020)

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Comparative bacteriological analysis of stored borehole water sources in Oyo town, Oyo State, Nigeria

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Water contamination is one of the leading causes of deaths and diseases worldwide, accounting for the deaths of more than 14,000 people daily, majority being children under 5 years old, therefore periodic monitoring of municipal water supplies are necessary. Comparative bacteriological analyses of twenty five samples of stored borehole water from five hostels in a tertiary institution in Oyo, Oyo State, was carried out using standard microbiological methods between March and April, 2018 to determine their bacteriological content. The hostels were: University Female Hostel (UFH), Diocese of Lagos West Hostel (DLW), Joseph Adetiloye Hostel (JAH), Diocese of Lagos Hostel (LAG) and Peter Jasper Akinola Hostel (IBD). A total of thirteen (13) different bacteria genera were isolated and identified as: *Aeromonas* (17%); *Escherichia* (4%); *Staphylococcus* (9%); *Pseudomonas* (9%); *Lactobacillus* (13%); *Corynebacterium* (27%); *Micrococcus* (6%); *Streptococcus* (3%); *Serratia* (4%); *Klebsiella* (1%); *Citrobacter* (3%); *Shigella* (3%); and *Salmonella* (1%). The total viable count ranged from 5.0×10^3 cfu/mL (IBD) to 1.23×10^5 cfu/mL (DLW). Total coliform bacteria counts of the stored water ranged from 0 to 28 MPN/100 mL. The bacteria load and presence of coliforms and pathogenic organisms raised grave concerns as WHO permissible limits for total viable count and coliforms are 100 cfu/mL and 0 MPN/100 mL, respectively. The organisms isolated are of public health significance as ingestion of water contaminated by them could result in gastroenteritis, especially *Escherichia* that indicates possible fecal contamination.

Key words: Bacteriological investigations, pathogens, groundwater, water quality, Oyo.

INTRODUCTION

The importance of water to human existence and ecological sustainability cannot be overrated as it is essential for life. It is found in virtually all living cells and is paramount to life. Although a human can do without food for up to twenty eight days, man cannot go without water for three days (Ukpong et al., 2013; Akin-Osanaiye et al., 2018).

Studies have proven that over one billion people in the world lack access to safe drinking water and about 2.5 billion people do not have access to adequate sanitation services at all (Tar et al., 2009). In developing countries such as Nigeria, clean pipe borne water availability is not

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available in almost all the states. Due to the inability of Government to meet the ever increasing water demand, people resort to ground water sources such as shallow wells and boreholes as alternative water resources (LAWMA, 2000). Natural groundwater is usually of good quality but this can deteriorate due to inadequate source of protection and poor resource management (Sadiya et al., 2018).

Groundwater is found beneath the earth's surface where it collects in voids of rocks and soil and it forms the ultimate source of water for springs, wells and boreholes. A borehole is a hydraulic structure which allows the withdrawal of water from an aquifer or groundwater resource (NWRI, 1997). Borehole water serves as the major source of drinking water in the local population of Nigeria (Akpoveta et al., 2011). Unfortunately, borehole water is not entirely pure and its purity depends on the geological conditions of the soil and in particular anthropogenic activities in the area which include: improper waste disposal, close proximity of septic to groundwater supply and leachate from landfills and dumpsites often polluting groundwater supply, thereby resulting in the transmission of bacteria and diseases (Boutaleb et al., 2008; Onwughara, et al., 2010).

Collected and stored borehole water microbial contamination is caused not only by the collection and use of faecally contaminated water that was not safe, to begin with, but also by the contamination of water (that was microbiologically safe initially), during storage. Unhygienic and imperfectly protected (poorly covered or open) water collection and containers for storage, unhealthy means of dispensing water from storage containers, including faecally contaminated dippers, hands, tools, lack of protection against vectors (flies, cockroaches, rodents, etc.) and inadequate cleaning of storage container to prevent biofilm formation and accumulation of sediments and pathogens, all are factors contributing to this problem (Steiner et al., 2006; Onwughara et al., 2010; Akpoveta et al., 2011).

Microorganisms play an important role in water quality and the microorganisms that are concerned with water borne diseases are *Salmonella* species, *Shigella* species, *Escherichia coli* and *Vibrio cholera.* The presence of faecal coliforms of *Escherichia coli* and those listed earlier are indicators of contaminated water (Adetunde and Glover, 2010).

Globally, water related diseases remain a major health concern. 1.8 million children die every year from waterborne diseases (that is to say 1 every 15 s). Worldwide, waterborne diseases are the most implicated killers of children under five years old and more people die annually from unsafe water than from violence (including war). Unsafe or inadequate water, sanitation, and hygiene account for 3.1% of all deaths worldwide. Unsafe water causes 4 billion cases of diarrhea each year, and results in 2.2 million deaths, mostly of children under five; a child dying every 15 s (UNESCO, 2017; WHO, 2019; Denchak, 2018).

The objectives of this study are therefore to determine the bacteriological enumeration, investigate the bacteriological content and compare bacteriological patterns of stored borehole water gotten from five different hostels water supplies in a tertiary institution within Oyo town of Oyo State, Nigeria.

MATERIALS AND METHODS

Study area

The study was carried out in a tertiary institution in Oyo, Oyo State, Nigeria. The institution is located on the Oyo-Ogbomoso road in Atiba Local Government Area (LGA) of Oyo State between longitude 3.9351°E and latitude 7.8371°N. The campus can be reached within 1 h by road from Ibadan and Ogbomoso, in 1.5 h from Ilorin and Ile-Ife and in 2.5 h from Lagos. The institution has five hostels, in which two (2) are allocated to females and three (3) to males. The five hostels are:

(a) University Female Hostel (UFH);
(b) Diocese of Lagos West Female Hostel (DLW);
(c) Joseph Adetiloye Hostel (JAH);
(d) Diocese of Lagos Hostel (LAG);
(e) Peter Jasper Akinola / Ibadan Hostel (IBD);

Each of the hostels has a source of borehole from which water is pumped into plastic storage tanks and then to an overhead tank. The hostel residents make use of the stored water for different purposes including drinking, washing and bathing. The water content of the tanks is used up daily due to the population of residents.

Sample collection

Water samples were collected from five (5) different storage tanks, holding water, pumped from individual boreholes of the hostels. The water samples were collected weekly (on Mondays) between the months of March and April 2018 which is a peak period in the use of the hostels. A total of five (5) samples were collected from each storage tank on five respective sampling trips which gave rise to twenty five (25) water samples in all. The average sampling time was 8 am each sampling day. Table 1 shows the description of the sampling areas with their geographical location. Samples were collected into 20 mL sterile sample bottles, screw capped and labeled. Samples were collected and transported using ice bag immediately to the laboratory for analysis.

Media used

Media for the multiple tube fermentation and plate counts were prepared according to the manufacturer's instructions. The media used were Nutrient Agar (Himedia), MacConkey Agar (Himedia), Lactose broth (Lab M), and Eosin Methylene Blue (Himedia) agar. All were prepared according to the manufacturer's instruction and sterilized in an autoclave at 121°C for 15 min.

Bacteriological analysis of water

Using the pour plate method, total heterotrophic bacteria in the water samples were obtained (Dhawale and LaMaster, 2003). Serial

Table 1. Sampling area description.

Sampling location	Identification code	Geographical location
University Female Hostel	UFH	$7°51^{1}47^{11}$N $3°56^{1}83^{11}$E
Diocese of Lagos West Hostel	DLW	$7°51^{1}50^{11}$N $3°56^{1}43^{11}$E
Joseph Adetiloye Hostel	JAH	$7°50^{1}50^{11}$N $3°56^{1}86^{11}$E
Peter Jasper Akinola / Ibadan Hostel	IBD	$7°50^{1}49^{11}$N $3°56^{1}48^{11}$E
Diocese of Lagos Hostel	LAG	$7°50^{1}48^{11}$N $3°56^{1}50^{11}$E

dilution was carried out and 1 mL aliquots of dilutions of 10^{-1} and 10^{-3} of each representative sample were inoculated into sterile Petri dishes. 10 mL of molten Nutrient agar (NA) was then introduced in the Petri dish over the samples. These were then swirled to attain even distribution and incubated at 37°C for 24 h. Petri-dishes from dilutions containing between 30 and 300 discrete colonies were counted and was made in cfu/mL (colony forming unit) (APHA, 2017). The colony forming units per millimeter (cfu/mL) was calculated by dividing the average number of colonies per dilution with the dilution factor.

A sterile inoculating loop was ascetically used to pick a loopful of each water sample. This was then streaked across the already set solid agar surface using the quadrant method of streaking. The inoculating loop was flamed between streaks and eventually after use; the plates were incubated at 37°C for 24 h.

Enumeration of total coliform bacteria

Multiple tube fermentation test

Multiple tube fermentation tests were conducted to enumerate total and faecal coliform (APHA, 2017). Total coliform count was determined with the aid of the three tube assay of the Most Probable Number (MPN) method.

Presumptive test

Presumptive coliform test was carried out using lactose broth. The first set of the three tubes had sterile 10 mL double strength lactose broth (DSLB) and the second and third sets had 10 mL single strength lactose broth (SSLB). Durham tubes were inserted in test tubes prior to sterilization. The three sets of the tubes received 1, 0.1 and 0.01 mL of water samples using sterile pipettes. All tubes were then incubated at 37°C for 24 to 48 h for estimation of total coliforms and examined afterwards for acid and gas production. The MPN was then determined from the MPN table for the three sets of tube (APHA, 2017).

Confirmed test

A loopful of culture was transferred from a positive tube from presumptive test into a tube of Lactose broth with Durham tube to carry out Confirmed test. The tubes were incubated at 37°C for 24 to 48 h for total coliform and 44.5 for faecal coliforms and observed for gas production.

Completed test

For the completed test a loopful of broth from a positive tube is streaked on Eosine Methylene Blue (EMB) agar plate for pure colonies. The plates were incubated at 37°C for 24 to 48 h. Colonies developing on EMB agar were further identified. Colonies with green metallic sheen were confirmed to be faecal coliform bacteria with rods shape.

Organisms observed with different morphology (mixed growth) were sub-cultured on Nutrient agar and Eosin Methylene Blue agar and incubated at 37°C for 24 h to get pure cultures.

After development of bacterial growth colony on the agar surface, cultural characteristics of the isolates on different solid agar were examined. Growth characteristics including colonial morphology, color pigmentation, form, deviation, margin, surface, and optical characters were recorded, following Bergey's manual of systematic bacteriology (Ryan and Ray, 2008).

Biochemical tests for identification of isolates

Biochemical tests carried out for identification of isolates were: Gram stain, Endospore test, Catalase test, oxidase, Sugar fermentation (TSI), Hydrogen sulphide, motility, Indole test; and Mannitol fermentation test.

RESULTS

The total heterotrophic counts of bacteria from stored borehole water samples of the various hostel ranged from 5.0×10^3 (IBD) to 1.23×10^5 cfu/mL (DLW) while the total coliform count of the stored water samples ranged from 0 (IBD) to 28 (UFH) MPN/100 mL (Table 2). Only Ibadan Hostel recorded 0 total coliform counts all through the study, next in line was Lagos Hostel which recorded low coliform counts on only 2 trips out of 5 UFH; however, had the highest number of coliform counts on four trips out of five as shown in Table 2. A total of 77 bacteria isolates were obtained in this study (Tables 3 to 7), 32 (41.6%) were Gram negative, while 45 of them (58.4%) were Gram positive bacteria. The most number of organisms were isolated from UFH, followed by LAG, next was JAH, then DLW and IBD had the least number of isolates (Tables 3 to 7). The 77 organisms isolated belonged to only 13 different bacterial genera which were characterized and identified as: *Corynebacterium (*27%), *Aeromonas* (17%), *Lactobacillus* (13%), *Escherichia* (4%), *Psuedomonas (*9%), *Staphylococcus* (9%), *Micrococcus* (6%), *Serratia* (4%), *Citrobacter* (3%), *Shigella* (3%), *Streptococcus* (3%), *Klebsiella* (1%), and *Salmonella* (1%) as shown in Figure 1. Table 8 shows a comparative representation of the different isolates from the hostels and reveals that UFH hostel had the most number of isolates, while LAG hostel had the least

Table 2. Total heterotrophic bacterial count and coliform count from the five hostels.

Hostel	Sample trip	Total cfu/mL	Total coliform count (MPN)/100 mL
JAH		3.6×10^4	17
UFH		5.7×10^4	21
LAG	1	3.2×10^4	-
DLW		8.3×10^4	24
IBD		4.1×10^4	-
JAH		2.3×10^4	21
UFH		1.16×10^5	28
LAG	2	3.1×10^4	2.8
DLW		4.3×10^4	24
IBD		2.3×10^4	-
JAH		2.1×10^4	17
UFH		8.6×10^4	24
LAG	3	3.4×10^4	-
DLW		1.23×10^5	17
IBD		4.7×10^4	-
JAH		1.4×10^4	21
UFH		1.6×10^4	28
LAG	4	2.0×10^4	-
DLW		2.5×10^4	17
IBD		5.0×10^3	-
JAH		4.1×10^4	24
UFH		2.8×10^4	28
LAG	5	5.2×10^4	8
DLW		1.96×10^5	21
IBD		1.18×10^5	-

UFH - University Female Hostel; DLW - Diocese of Lagos West Hostel; JAH - Joseph Adetiloye Hostel; LAG - Diocese of Lagos Hostel; IBD - Ibadan / Peter Jasper Akinola Hostel.

number of organisms.

Data shown in Table 8 further reveal the following occurrence of organisms according to site of isolation: *Aeromonas* species and *Corynebacterium* species were isolated from all five hostels studied (UFH, DLW, JAH, LAG and IBD). *Staphylococcus* species were isolated from UFH, JAH, LAG and IBD hostels, *Pseudomonas* species from UFH, DLW, JAH, and IBD hostels. *Lactobacillus* species found occurrence in UFH, DLW, and JAH hostels. *E. coli* and *Streptococcus* species were isolated from UFH and JAH hostels, *Micrococcus* species from UFH and DLW hostels, *Serratia* species from UFH and IBD hostels, *Shigella* species from LAG and IBD hostels. *Klebsiella* and *Citrobacter* species each found occurrence only in JAH hostel, while *Salmonella* species was isolated only from LAG hostel.

DISCUSSION

The total heterotrophic bacteria count from the five hostels indicated that none of the stored water samples fell within the 100 cfu/mL limit allowed by WHO (2006) for potable water. The high viable bacteria count of the stored water can be attributed to lack of water treatment and hygienic care (for example, washing) of the storage tanks. Most of the sampled waters recorded total coliform counts exceeding the WHO recommended standards which stipulate that total coliform counts should not exceed 1-3/100 mL of potable water and 0/100 mL of thermotolerant coliforms (WHO, 2006) and SON standards that recommend 0/100 mL of coliforms in portable water. Coliforms are indicator organisms and their presence is indicative of other disease causing organisms in the sampled water (Bello et al., 2013). The total coliform count obtained implies contamination, possibly from piping leakages within the water system network, unsanitary conditions or groundwater contamination.

Three members of the coliform bacteria group were isolated in this study (*Escherichia*, *Citrobacter* and *Klebsiella*). Coliforms are important markers for bacteriological water quality as they are established

Table 3. Biochemical characteristics and identification of isolates from university female hostel.

Isolate code	Gram staining	Morphology	Catalase	Oxidase	Indole	Motility	H_2S	Lactose	Sucrose	Glucose	Gas	Spore forming	Mannitol	Yellow pigment	Organism
UFH 1a	-	Rod	+	+	-	-	-	-	-	-	-	-	-	-	*Aeromonas* spp.
UFH 1b	-	Rod	+	+	-	+	-	+	+	+	+	-	-	-	*Serratia* spp.
UFH 1c	+	Rod	+	+	-	-	+	+	+	+	+	-	-	-	*Corynebacterium* spp.
UFH 1d	+	Rod	-	+	-	-	+	+	+	+	-	-	-	-	*Lactobacillus* spp.
UFH 1e	+	Rod	+	+	-	+	-	+	+	+	+	-	-	-	*Corynebacterium* spp.
UFH 1f	-	Short Rod	+	+	-	-	-	-	-	-	-	-	-	-	*Pseudomonas* spp.
UFH 2a	+	Cocci	+	+	-	+	-	+	+	+	+	-	+	+	*Staphylococcus* spp.
UFH 2b	+	Cocci	+	-	-	+	-	-	-	-	-	-	+	+	*Micrococcus* spp.
UFH 2c	+	Cocci	-	-	+	+	-	+	+	+	+	-	+	+	*Streptococcus* spp.
UFH 2d	+	Rod	+	+	-	+	+	+	+	+	+	-	-	-	*Corynebacterium* spp.
UFH 3a	-	Short Rod	+	+	-	-	-	+	+	+	-	-	-	-	*Aeromonas* spp.
UFH 3b	+	Short Rod	-	+	-	+	-	+	+	+	+	-	-	-	*Lactobacillus* spp.
UFH 3c	-	Rod	+	+	-	-	-	+	+	+	-	-	-	-	*Aeromonas* spp.
UFH 3d	-	Rod	+	+	-	-	+	-	-	-	-	-	-	-	*Pseudomonas* spp.
UFH 4a	-	Short Rod	+	+	+	-	-	+	+	+	-	-	-	-	*Escherichia coli*
UFH 4b	+	Rod	-	+	-	+	-	+	+	+	+	-	-	-	*Lactobacillus* spp.
UFH 4c	+	Rod	+	+	-	+	-	+	+	+	-	-	-	-	*Corynebacterium* spp.
UFH 5a	+	Rod	+	+	-	+	-	-	-	-	-	-	-	-	*Aeromonas* spp.
UFH 5b	-	Rod	+	+	-	-	+	-	-	-	-	-	-	-	*Pseudomonas* spp.
UFH 5c	-	Short Rod	+	-	-	+	-	-	-	-	-	-	-	-	*Serratia* spp.

UFH-University Female Hostel, 1-Sample trip 1, 2-Sample trip 2, 3-Sample trip 3, 4-Sample trip 4, 5-Sample trip 5; a, b, c, d, e, respective isolates; spp.-Species, H_2S-Hydrogen Sulphide.

causes for human gastroenteritis and their presence in potable water makes it unsafe for consumption. The most implicated coliform, *E. coli* indicates recent faecal contamination. *E. coli*, a normal intestinal gut flora, is found in the gut of humans and animals, where it is harmless (WHO, 2017). However, in other parts of the body, *E. coli* can cause serious disease. Two particular pathogenic serotypes of *E. coli* namely *E. coli* O157:H7 and *E. coli* O104:H4 are known causes of diseases in humans (Ateba and Bezeuidenhout, 2008). A severe outbreak of illness caused by *E. coli* O157:H7 was recorded in Walkerton community, Ontario, Canada resulting in 7 deaths and over 2300 cases of illness (Aboh et al., 2015). Coliforms have been isolated from soil and water and their presence in the samples tested suggests possible breakage of the piping system. Moreover, the presence of coliforms in tested water samples implies a failure to meet up with WHO standards.

Worldwide, 80 to 165 million cases of infection from *Shigella* are reported, causing 600,000 deaths annually largely in developing countries, the most vulnerable group being children below 10 years (WHO, 2017), which makes the presence of *Shigella* unacceptable among others. The incidence of high numbers of *Pseudomonas* in potable water often elicits foul odour, taste, and high turbidity levels. *Salmonella* also isolated in this study clinically manifests in gastroenteritis in humans and one of its serotype-*typhi* causes typhoid fever with devastating public health implications (WHO, 2017). These organisms are commonly found in the environment with some species being host specific particularly in animals

Table 4. Biochemical characteristics and identification of isolates from Joseph Adetiloye Hostel.

Isolate code	Gram staining	Morphology	Catalase	Oxidase	Indole	Motility	H_2S	Lactose	Sucrose	Glucose	Gas	Spore forming	Mannitol	Yellow	Organism
JAH 1a	+	Rod	+	+	-	+	-	+	+	+	+	-	-	-	*Lactobacillus fermentii*
JAH 1b	+	Rod	+	+	-	-	-	+	+	-	-	-	-	-	*Corynebacterium* spp.
JAH 1c	-	Rod	+	+	-	-	-	-	-	-	-	-	-	-	*Pseudomonas* spp.
JAH 2a	+	Cocci	-	-	-	-	-	-	-	-	-	-	+	+	*Streptococcus* spp.
JAH 2b	+	Cocci	+	+	+	+	-	-	-	-	-	-	+	+	*Stapylococcus* spp.
JAH 2c	+	Rod	+	-	-	+	-	+	+	+	+	-	-	-	*Lactobacillus fermentii*
JAH 2d	+	Rod	-	+	-	-	-	+	+	+	+	-	-	-	*Lactobacillus fermentii*
JAH 2f	+	Rod	-	+	+	+	+	+	+	+	+	-	-	-	*Lactobacillus fermentii*
JAH 3a	+	Rod	+	+	-	-	-	+	+	+	+	-	-	-	*Klebsiella* spp.
JAH 3b	+	Rod	+	+	-	+	-	-	-	+	-	-	-	-	*Corynebacterium* spp.
JAH 3c	-	Rod	+	+	+	-	-	+	+	+	+	-	-	-	*Escherichia coli*
JAH 3c	-	Rod	+	+	-	-	-	+	+	-	-	-	-	-	*Pseudomonas* spp.
JAH 4a	-	Rod	+	+	-	+	+	+	+	+	+	-	-	-	*Aeromonas hydrophilic*
JAH 4b	-	Rod	+	+	-	+	+	+	+	+	+	-	-	-	*Citrobacter* spp.
JAH 4c	+	Rod	+	+	+	+	+	+	+	+	+	-	-	-	*Escherichia coli*
JAH 4d	-	Rod	+	+	-	-	-	+	+	+	-	-	-	-	*Aeromonas veronii*
JAH 5a	-	Rod	+	+	-	+	+	+	+	+	+	-	-	-	*Citrobacter* spp.

JAH-Joseph Adetiloye Hostel, 1-Sample trip 1, 2-Sample trip 2, 3-Sample trip 3, 4-Sample trip 4, 5-Sample trip 5; a,b,c,d,e, respective isolates; spp.-Species, H_2S-Hydrogen Sulphide.

and also humans. Their presence in tested samples suggest possible compromise of nearby sewage system, water piping leakages and They are opportunistic pathogens that are known to induce chronic diarrhea in humans when water polluted by them is ingested (Igbinosa et al., 2012; Pavan et al., 2013). There is a possibility that the storage tanks were not properly covered or covers may have depreciated due to weathering.

Most of the organisms isolated are important human pathogens associated with variety of infectious diseases and outbreaks including: gastroenteritis, typhoid fever, dysentery, cholera, urinary tract infection among others, especially perhaps questionable personal hygiene of the operators.

Micrococcus and *Aeromonas* are known water where such bacteria possess virulence factor genes (Orji et al., 2006; Uzoigwe and Agwa, 2012; Bello et al., 2013). They belong to the family of Enterobacteriaceae, and members of this family are generally spread via the faecal-oral route, and their presence in the samples indicates contamination of the water supplies. If such water is consumed without treatment, it poses grave health hazards to humans (WHO, 2006; SON, 2015).

This study also reveals disparities in organisms contaminants that easily proliferate in waters exposed to contaminated air, dust and where water holding vessels are not cleaned regularly. isolated from the different hostels. This indicates differing sanitary and environmental conditions of the storage tanks in the various hostels. It also implies that the contamination source is peculiar to prevailing conditions of the respective sampling sites.

Conclusion

This study isolated pathogenic organisms from stored borehole water supplies in a tertiary

Table 5. Biochemical characteristics and identification of isolates from Diocese of Lagos West Hostel.

Isolate code	Gram staining	Morphology	Catalase	Oxidase	Indole	Motility	H_2S	Lactose	Sucrose	Glucose	Gas	Spore forming	Mannitol	Yellow	Organism
DLW 1a	-	Short Rod	+	+	-	+	-	+	+	+	+	-	-	-	*Aeromonas veronii*
DLW 1b	+	Short Rod	+	-	-	-	-	+	+	+	+	-	-	-	*Corynebacterium* spp.
DLW 1c	+	Cocci	+	+	-	-	-	-	-	-	-	-	+	+	*Micrococcus* spp.
DLW 1d	-	Rod	+	+	-	+	+	-	-	-	-	-	-	-	*Pseudomonas* spp.
DLW 1e	+	Cocci	+	-	-	+	-	+	+	+	-	-	+	+	*Micrococcus* spp.
DLW 1f	+	Cocci	+	-	-	+	-	+	+	+	+	-	+	+	*Micrococcus* spp.
DLW 2a	+	Rod	-	+	-	+	+	+	+	+	+	-	-	-	*Lactobacillus fermentii*
DLW 2b	-	Rod	+	+	-	+	+	+	+	+	+	-	-	-	*Corynebacterium* spp.
DLW 2c	-	Rod	+	+	+	+	-	-	-	+	+	-	-	-	*Aermonas veronii*
DLW 2d	-	Short Rod	+	+	-	+	+	+	+	+	+	-	-	-	*Aeromonas hydrophila*
DLW 3b	+	Rod	+	-	+	+	-	-	-	-	-	-	-	-	*Corynebacterium* spp.
DLW 4a	-	Rod	-	+	-	+	-	+	+	+	-	-	-	-	*Lactobacillus delbrueckii*
DLW 4b	+	Rod	-	+	-	+	+	+	+	+	+	-	-	-	*Lactobacillus fermentii*
DLW 5a	-	Rod	+	+	-	+	-	+	+	+	-	-	-	-	*Aeromonas veronii*
DLW 5b	+	Cocci	+	+	-	-	-	+	+	+	+	-	+	+	*Micrococcus* spp.

DLW-Diocese of Lagos West Hostel; 1-Sample trip 1, 2-Sample trip 2, 3-Sample trip 3, 4-Sample trip 4, 5-Sample trip 5; a,b,c,d,e, respective isolates; spp.-Species, H_2S-Hydrogen Sulphide.

Table 6. Biochemical characteristics and identification of isolates from Peter Jasper Akinola Hostel (IBADAN).

Isolate code	Gram staining	Morphology	Catalase	Oxidase	Indole	Motility	H_2S	Lactose	Sucrose	Glucose	Gas	Spore forming	Mannitol	Yellow	Organism
IBD 1a	+	Rod	+	-	-	-	-	-	-	-	-	-	-	-	*Corynebacterium* spp.
IBD 1b	-	Rod	+	-	-	+	-	-	-	-	-	-	-	-	*Serratia liquefaciens*
IBD 2b	+	Cocci	+	-	-	-	-	-	-	-	-	-	+	+	*Staphylococcus* spp.
IBD 3a	+	Long Rod	+	-	-	+	-	+	+	+	-	-	-	-	*Corynebacterium* spp.
IBD 3b	-	Short Rod	+	+	-	+	+	+	+	+	+	-	-	-	*Aeromonas veronii*
IBD 4a	+	Cocci	+	+	+	+	-	+	+	+	+	-	+	+	*Staphylococcus* spp.
IBD 4c	-	Short Rod	+	+	-	+	-	+	+	+	+	-	-	-	*Pseudomonas* spp.
IBD 4d	+	Rod	+	+	-	+	-	+	+	+	+	-	-	-	*Corynebacterium* spp.
IBD 5a	-	Short Rod	-	-	-	-	-	-	-	-	-	-	-	-	*Shigella* spp.
IBD 5b	+	Rod	+	+	-	-	-	+	+	-	-	-	-	-	*Corynebacterium* spp.
IBD 5c	+	Rod	+	-	-	+	-	+	+	-	-	-	-	-	*Corynebacterium* spp.

IBD-Peter Jasper Akinola / Ibadan Hostel; 1-Sample trip 1, 2-Sample trip 2, 3-Sample trip 3, 4-Sample trip 4, 5-Sample trip 5; a,b,c,d,e, respective isolates; spp.-Species, H_2S-Hydrogen Sulphide.

Table 7. Biochemical characteristics and identification of isolates from Diocese of Lagos Hostel.

Isolate code	Gram staining	Morphology	Catalase	Oxidase	Indole	Motility	H_2S	Lactose	Sucrose	Glucose	Gas	Spore forming	Mannitol	Yellow	Organism
LAG 1a	+	Rod	+	+	-	-	-	+	+	+	-	-	-	-	*Corynebacterium* spp.
LAG 1b	+	Rod	+	+	-	+	-	+	+	+	-	-	-	-	*Corynebacterium* spp.
LAG 1c	+	Rod	+	+	-	+	-	-	-	-	-	-	-	-	*Corynebacterium* spp.
LAG 1d	+	Rod	+	+	-	+	-	-	-	-	-	-	-	-	*Corynebacterium* spp.
LAG 2a	+	Cocci	+	+	-	+	-	-	-	-	-	-	-	-	*Staphylococcus* spp.
LAG 2b	-	Rod	+	-	-	-	-	-	-	+	-	-	-	-	*Shigella* spp.
LAG 2c	-	Short Rod	+	-	+	+	+	+	+	+	-	-	-	-	*Samonella* spp.
LAG 2d	-	Rod	+	+	-	-	+	+	+	+	-	-	-	-	*Aeromonas hydrophila*
LAG 3a	-	Rod	+	+	-	+	+	+	+	+	+	-	-	-	*Aeromonas hydrophila*
LAG 3b	+	Long Rod	+	+	-	+	-	-	-	-	-	-	-	-	*Corynebacterium* spp.
LAG 4a	+	Cocci	+	+	-	-	-	-	-	-	-	-	+	+	*Staphylococcus* spp.
LAG 5a	-	Rod	+	-	-	+	+	+	+	+	+	-	-	-	*Aeromonas hydrophila*
LAG 5b	+	Cocci	+	+	-	+	-	+	+	+	+	-	+	+	*Staphylococcus* spp.
LAG 5c	+	Rod	+	+	-	+	-	-	-	-	-	-	-	-	*Corynebacterium* spp.
LAG 3b	+	Long Rod	+	+	-	+	-	-	-	-	-	-	-	-	*Corynebacterium* spp.
LAG 4a	+	Cocci	+	+	-	-	-	-	-	-	-	-	+	+	*Staphylococcus* spp.
LAG 5a	-	Rod	+	-	-	+	+	+	+	+	+	-	-	-	*Aeromonas hydrophila*
LAG 5b	+	Cocci	+	+	-	+	-	+	+	+	+	-	+	+	*Staphylococcus* spp.
LAG 5c	+	Rod	+	+	-	+	-	-	-	-	-	-	-	-	*Corynebacterium* spp.

LAG-Diocese of Lagos Hostel**,** 1-Sample trip 1, 2-Sample trip 2, 3-Sample trip 3, 4-Sample trip 4, 5-Sample trip 5; a,b,c,d,e, respective isolates; spp.-Species, H_2S-Hydrogen Sulphide.

institution. These findings prove that all the stored water tested failed to meet up with approved SON and WHO standards for portable water. Consumption of water from these supplies without treatment may pose serious health risks to the consumers.

Recommendations

There is a need to sensitize operators of boreholes and end users of the stored water on the importance of maintaining clean and hygienic environmental conditions around the borehole and water storage tanks to prevent contamination. Further investigation of the waste disposal system of hostels where coliforms were isolated from samples is necessary, together with proper monitoring of environmental conditions of the water systems in all hostels. Adequate water disinfection and treatment of

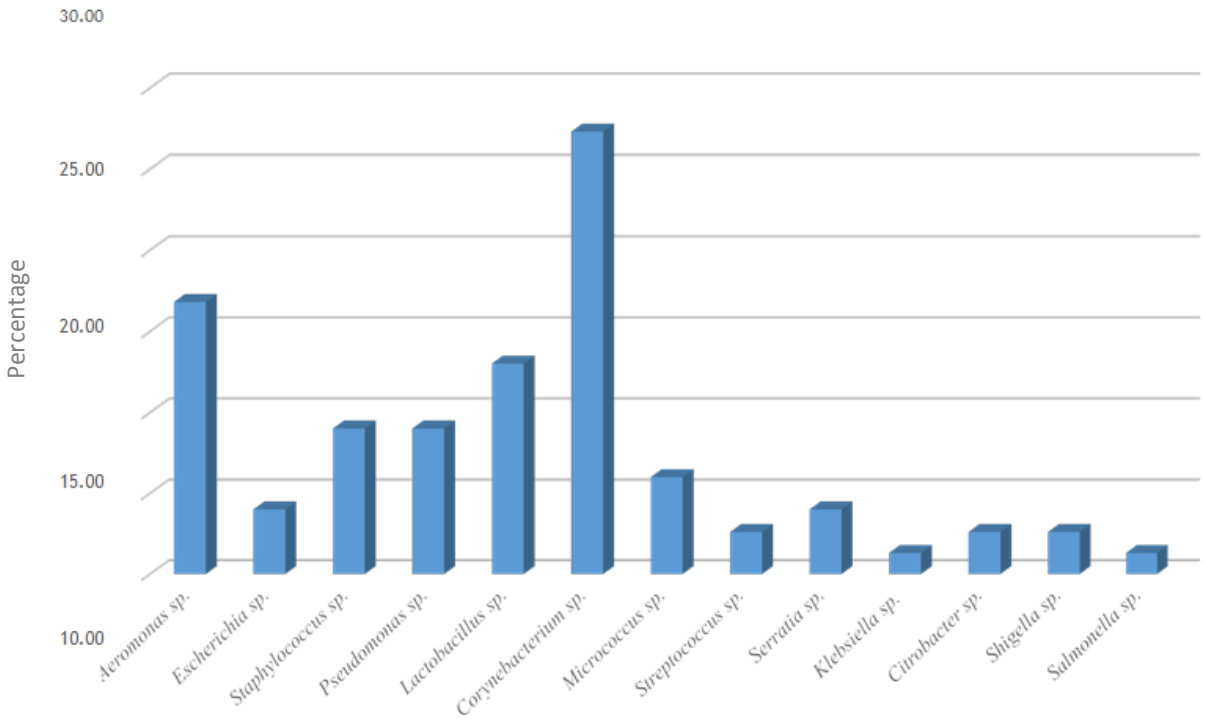

Figure 1. Frequency (%) of occurrence of bacteria isolated from stored borehole water.

Table 8. Comparative frequency of occurrence of organisms.

Isolate	UFH (%)	DLW (%)	JAH (%)	LAG (%)	IBD (%)
Aeromonas spp.	20	23.5	11.8	21	9
Escherichia coli.	5	AB	11.8	AB	AB
Staphylococcus spp.	5	AB	6	21	18
Pseudomonas spp.	15	6	11.8	AB	9
Lactobacillus spp.	15	17.6	23.5	AB	AB
Corynebacterium spp.	20	17.6	17.6	43	45
Micrococcus spp.	5	23.5	AB	AB	AB
Streptococcus spp.	5	AB	6	AB	AB
Serratia spp.	10	AB	AB	AB	9
Klebsiella spp.	AB	AB	6	AB	AB
Citrobacter spp.	AB	AB	11.8	AB	AB
Shigella spp.	AB	AB	AB	7	9
Salmonella spp.	AB	AB	AB	7	AB

AB-Absent, UFH-University Female Hostel, DLW-Diocese of Lagos West Hostel, JAH- Joseph Akintiloye Hostel, LAG-Diocese of Lagos Hostel, IB-Peter Jasper Akinola Hostel (Ibadan).

all storage water tanks is advocated to prevent any adverse effect to end users of the water supplies. Regular water system monitoring and analysis will be instrumental in ensuring water supplies fall within approved limits.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Aboh EA, Giwa FJ, Giwa A (2015). Microbiological assessment of well waters in Samaru, Zaria, Kaduna, State, Nigeria. Annals of African Media 14:32-38.

Adetunde LA, Glover RLK (2010). Bacteriological quality of borehole water used by students' of University for Development Studies, Navrongo Campus in Upper-East Region of Ghana. Current Research Journal of Biological Sciences 2(6):361-364.

Akin-Osanaiye BC, Mohammed SE, Echoki J (2018). Comparative analysis of pipe borne water and other sources of water in

Gwagwalada Area Council, Federal Capital Territory, Abuja, Nigeria. Journal of Biology and Genetic Research 4(1):2545-5710

Akpoveta OV, Okoh BE, Osakwe SA (2011). Quality assessment of borehole water used in the vicinities of Benin, Edo State and Agbor, Delta State of Nigeria. Current Research in Chemistry, 3(1):62-67.

American Public Health Association (APHA) (2017). Standard Methods for the Examination of Water and Wastewater, 21st Edn., APHA, Washington DC. Available online at https://www.awwa.org/Store/Standard-Methods-for-the-Examination-of-Water-and-Wastewater-23rd-Edition/ProductDetail/65266295

Ateba CN, Bezuidenhout CC (2008). Characterisation of *Escherichia coli* O157 strains from humans, cattle and pigs in the North-West Province, South Africa. International Journal of Food Microbiology 128(2):181-8.

Bello OO, Osho A, Bankole SA, Bello TK (2013). Bacteriological and physicochemical analyses of borehole and well water sources in Ijebu-Ode, Southwestern Nigeria. International Journal of Pharmacy and Biological Sciences 8:18-25.

Boutaleb S, Boualoul M, Bouchaou L, Oudra M (2008). Application of remote sensing and surface geophysics for groundwater prospecting in a hard terrain, Morocco. In S.Adelana and A.MacDonald (Ed.) Applied groundwater studies - IAH selected papers on hydrogeology. Taylor and Francis Group, London. 13:215-226.

Denchak M (2018). Water pollution: Everything you need to know. Retrieved online on 10:11:18 from: https://www.nrdc.org/stories/water-pollution-everything-you-need-know

Dhawale S, LaMaster A (2003). Microbiology Laboratory manual. The mcHill Company. Inc. USA. p. 226

Igbinosa IH, Igumbor EU, Aghdasi F, Tom M, Okoh AI (2012). Emerging *Aeromonas* species infections and their significance in public health. Scientific World Journal 2012:625023.

Lagos State Waste Management Authority (LAWMA) (2000). Management of landfill wastes: The Journey So Far. L.E.P.A. Handbook.

National Water Research Institute, NWRI (1997). Training Guide for Basic Water Treatment Operations. Kaduna. National Water Resources Institute. 1.

Onwughara IN, Nnorom IC, Kanno OC (2010): Issues of roadside disposal habit of municipal solid waste, environmental impacts and implementation of sound management practices in developing country: Nigeria. International Journal of Environmental Science and Development 1(5):409-417.

Orji MU, Ezenwaje EE, Anyaegbunam BC (2006). Spatial appraisal of shallow well water pollution in Awka, Nigeria. Nigerian Journal of Microbiology 20(3):1389-1384.

Pavan KP, Raghuveer YP, Shiva SA (2013). Identification of opportunistic pathogenic bacteria in drinking water samples of different rural health centers and their clinical impacts on humans. BioMed Research International 2013(2):348250.

Ryan KJ, Ray CG (2008). Sherris Medical Microbiology, 4th edition. McGraw Hill pp. 370-380.

Sadiya A, Chukwuma CO, Olatunbosun OA, Onyinye FN (2018). Comparative study of the physicochemical and bacteriological qualities of some drinking water sources in Abuja, Nigeria. Global Journal of Pure and Applied Sciences 24: 91-98.

Standards Organization of Nigeria (SON) (2015). Nigerian Standard for Drinking Water Quality NIS 554:2015. Retrieved online on 15:03:19 from: https://africacheck.org/wp-content/uploads/2018/06/Nigerian-Standard-for-Drinking-Water-Quality-NIS-554-2015.pdf

Steiner TS, Samie A, Guerrant RL (2006). Infectious diarrhea: new pathogens and new challenges in developed and developing areas. Clinical Infectious Diseases 43:408-410.

Tar A, Eneji I, Ande S, Oketunde F, Ande S, Shaaton R (2009). An assessment of heavy metals loading in River Benue in the Makurdi Metropolitan Area in Central Nigeria. Nigerian Journal of Chemical Society of Nigeria 34:56-62.

Ukpong EC, Ogarekpe NM, Bejor ES (2013). Comparative analysis of water quality in hand dug well and borehole in Calabar South Local Government Area in Nigeria. The International Journal of Engineering and Science 2(8):95-101.

United Nations Educational, Scientific and Cultural Organization (UNESCO) (2017). Facts and Figures. Wastewater: The untapped Resource. The United Nations World Water Development Report 2017. Retrieved online on 30:03:2019 from: http://unesdoc.unesco.org/images/0024/002475/247553e.pdf

Uzoigwe CI, Agwa OK (2012). Microbiological quality of water collected from boreholes sited near refuse dumpsites in Port Harcourt, Nigeria. African Journal of Biotechnology 11(13):3135-3139.

World Health Organization (WHO) (2006). Guidelines for Drinking Water Quality. Geneva. Addendum to the 3rd Vol. 1 Recommendations. World Health Organization 1:23-48.

World Health Organization (WHO) (2017). Guidelines for Drinking-Water Quality: Fourth Edition Incorporating the First Addendum. Geneva: World Health Organization; 2017. Retrieved online from: https://www.ncbi.nlm.nih.gov/books/NBK442376/

World Health Organization (WHO) (2019). Sanitation. Retrieved on 28:10:18 from: http://www.who.int/news-room/fact-sheets/detail/sanitation.

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Antimicrobial susceptibility of Flavobacteriaceae isolates from Nile Tilapia (*Oreochromis niloticus*) in Tanzania

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This study aimed to assess antimicrobial susceptibility of members of the family Flavobacteriaceae isolated from Nile tilapia (*Oreochromis niloticus*). Antimicrobial susceptibility of 67 Flavobacteriaceae isolates originating mainly from ponds and Lake Victoria against 19 antimicrobial agents was determined by the broth micro dilution method. Overall, most isolates were susceptible to enrofloxacin (97%; MIC_{90} 2 µg/ml) followed by novobiocin (85%, MIC_{90}, 4 µg/ml) and the aminoglycoside streptomycin (85%; MIC_{90}, 128 µg/ml). Some isolates were also susceptible towards trimethoprim/sulfamethoxazole (77.6%), neomycin and florfenicol both at 62.7%. Susceptibility levels were low for tylosin tartrate (32.8%), clindamycin and sulphathiazole both at (23.9%), ceftiofur (6%), spectinomycin (6%) and tetracyclines/oxtetracyclines (4.5%). In contrast, β-Lactams (amoxicillin, penicillin), gentamycin and erythromycin exhibited very poor activity against Flavobacteriaceae isolates. The extent of antimicrobial susceptibility did not vary significantly among isolates from farmed and wild fish isolates (P > 0.01). The highest Multiple Antimicrobial Resistance (MAR) index was observed in *Chryseobacterium indologenes* (0.89) and the lowest in *Chaetoderma indicum* isolates (0.32). Our results indicate that most of Flavobacteriaceae isolates are multidrug resistance, and this may be associated with intrinsic resistance mechanisms to a broad range of antimicrobial agents. However, the need remains to carryout in-depth study to understand better the underlying genetic mechanisms given that the magnitude and trend for susceptibility was comparable between isolates from aquaculture and fisheries. The findings from this study give us insight into appropriate choice of antimicrobial agents for effective treatment of infections caused by these isolates.

Key words: Aquaculture, Fisheries, Intrinsic resistance, minimum inhibitory concentrations, ponds, Lake Victoria.

INTRODUCTION

The genera *Flavobacterium* and *Chryseobacterium* belong to the family Flavobacteriaceae and are widely distributed in various environments, including soil, freshwater and saltwater ecosystems (Kumru et al., 2017; McBride, 2014). Several species within the Flavobacteriaceae are regarded as opportunistic

pathogens, yet with potential to cause diseases in a wide variety of organisms, including plants, fish and humans (Bernardet and Bowman, 2011; Bernardet and Nakagawa, 2006; Loch and Faisal, 2015). Typically, many of these bacteria cause diseases in fish when water temperatures are relatively high. Excessive organic matter in ponds and high stocking density are among other contributing factors to diseases that consequently lead to economic losses (Loch et al., 2013). In addition, these bacteria have also been associated with nosocomial infections and septicaemia in humans (Loch et al., 2013; Ratnamani, 2013).

Fish farming has grown worldwide as a source of high quality protein and employment to skilled and unskilled workers. In Tanzania, aquaculture is dominated by pond culture of Nile tilapia (*Oreochromis niloticus*). As aquaculture has developed, a range of bacterial diseases have been encountered (Pathmalal et al., 2018). These diseases are considered as critical limiting factors in this industry, where studies have been conducted and efforts has been given to therapeutic and prophylactic use of antimicrobial agents as control measures of bacterial diseases (Cabello et al., 2006). Many European countries are governed by legislation and regulation on the use of antimicrobial agents in aquaculture (Cabello et al., 2013). However, guidelines for the correct and prudent use of antimicrobial agents in aquaculture have yet to be developed in most of the African countries. There is no list of licensed antimicrobials for use in aquaculture in Tanzania. Previous work has shown that extensive use of antimicrobials in aquaculture leads to resistance across the entire microbial water ecosystem (Schmidt et al., 2000, Watts et al., 2017). In the family Flavobacteriaceae, some antimicrobial resistance has been associated with the presence of plasmids or mutations in specific resistance determinants (Izumi et al., 2004, Madsen et al., 2000). However, other studies have associated antimicrobial resistance in Flavobacteriaceae with intrinsic resistance to a wide range of antimicrobial agents through mechanisms like restricted outer membrane permeability, efflux systems that pump antimicrobials out of the cell and production of antimicrobial-inactivating enzymes such as β-lactamases (Clark et al., 2009; Henríquez-Núñez et al., 2012; Michel et al., 2005).

The occurrence of resistant bacteria to one or more antimicrobials have been reported not only in pathogenic fish bacteria but also among opportunistic bacteria, even in the absence of selective antimicrobial pressure in the aquaculture environment (Gufe et al., 2019; Jensen et al., 2008). In Tanzania, the nutritional benefits of fish consumption have a positive link to increased food security, and the aquaculture sector has the potential to play a significant role in this aspect. However, a sustainable fish production has to be secured, including the development of control measures to reduce the spread of resistant bacteria in the aquaculture environments. As very few data are currently available, the aim of this study was to assess the antimicrobial susceptibility of Flavobacteriaceae isolates from Nile tilapia sampled directly from fish farms as well as natural lacustrine environment in Tanzania. Findings from this study would contribute to our understanding of antimicrobial susceptibility in the Flavobacteriaceae; provide basic knowledge needed in implementation of biosecurity measures in fish farming and give a background to treatment of farmed fish if necessary.

MATERIALS AND METHODS

Bacterial isolates from Tanzania

A total of 67 Flavobacteriaceae isolates previously recovered from 40 Nile tilapia farmed in the Morogoro region, and from batch of 21 wild Nile tilapia collected from natural environment in Lake Victoria in Mwanza region. Apparently healthy fish were sampled between November 2015 and May 2016, isolation and identification of the bacteria were performed as previously described in our earlier work (Mwega et al., 2018). Briefly, all isolates were grown on modified Anacker and Ordal agar - AOA (Pilarski et al., 2008). Identification of isolates based on colony morphology and biochemical testing, and was confirmed by 16S rRNA gene sequencing (Darwish et al., 2004). All isolates used in this study were designated with the organism code based on sampling location as shown in Table 3.

Antimicrobial susceptibility tests

Antimicrobial susceptibility to 19 antimicrobials commonly used in veterinary medicine and aquaculture in most developed countries was assessed for all isolates using Trek Sensititre Avian susceptibility plates (Trek Diagnostic Systems, Cleveland, OH) as shown in Table 1. These test plates were 96-well, dry-form that contained twofold serial dilutions of the antimicrobial agents listed in Table 1. Briefly, ten microliters of the bacteria suspension were transferred into a tube containing 11 ml of Sensititre MH broth to give an inoculum of 1×10^5 CFU/ml. The broth was poured into a sterile seed trough and individual wells inoculated with 50 µl using a multi-channel pipette. Inoculated plates were sealed and incubated aerobically ATCC 25922 and *Aeromonas salmonicida* NCIMB 1102 were also included in parallel in all testing.

Data management and analysis

In the absence of published resistance breakpoints for Flavobacteriaceae, quantitative interpretation of minimum inhibition concentration (MIC) results was based on CLSI guidelines (CLSI, 2014) with the minor modifications as previously recommended (Akinbowale et al., 2006). Minimum inhibition concentration was recorded as the lowest concentration of antimicrobial that inhibited

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Table 1. Antimicrobial susceptibility of Flavobacteriaceae isolates (N = 67) to a spectrum of 19 antimicrobial agents.

Classes	Antimicrobial agents	Range (µg/ml)	MIC50	MIC90	n (%) of susceptible isolates
β-Lactams	Amoxicillin	0.25-16	> 16	>16	0.00
	Penicillin	0.06-8	> 8	> 8	0.00
	Ceftiofur	0.25-4	4	> 4	4 (6%)
Aminoglycosides	Gentamicin	0.5-8	> 8	> 8	0.00
	Spectinomycin	8.0 - 64	8	> 64	4 (6%)
	Streptomycin	8-1024	32	128	57 (85%)
	Neomycin	2.0 - 32	16	> 32	42 (62.6%)
Sulfonamides	SXT	0.5/9.5–2/38	< 0.5/9.5	1.0/19	52 (77.6%)
	Sulphathiazole	32-256	128	> 256	16 (23.9%)
	Sulphadimethoxine	32-256	> 256	> 256	0.00
Tetracyclines	Tetracycline	0.25-8	1	> 8	3 (4.5%)
	Oxytetracycline	0.25-8	1	> 8	3 (4.5%)
Macrolides	Tylosin tartrate	2.5 - 20	5	> 20	22 (32.8%)
	Erythromycin	0.12-4	> 4	> 4	0.00
Fluoroquinolones	Enrofloxacin	0.12-2	0.25	1	65 (97%)
Phenicols	Florfenicol	1.0 - 8	2	> 8	42 (62.7%)
Coumarins	Novobiocin	0.5-4	< 1	4	57 (85%)
Lincomycins	Clindamycin	0.5- 4	2	> 4	16 (23.9%)

SXT – Trimethoprim/ sulfamethoxazole.

visible growth. The MIC50 and MIC90 represent the MIC value at which ≥ 50% and ≥ 90% of the isolates were inhibited respectively. A multiple antimicrobial resistance (MAR) index was then determined for each isolate by dividing the number of antimicrobials to which an isolate was resistant with the total number of antimicrobials tested. The MAR index analysis was used to group the different sources from which the bacteria were recovered using the frequency of antimicrobial resistance. Isolates with MAR index < 0.2 were considered as isolates recovered from low-risk sources of contamination while isolates with MAR index > 0.2 were considered recovered from high- risk sources (Samuel et al., 2011; Tambekar et al., 2006).

RESULTS

Antimicrobial susceptibility of Flavobacteriaceae isolates

The *in vitro* activities of the 19 antimicrobial agents tested against the Flavobacteriaceae isolates are summarized in Table 1, showing both the MIC range and the MIC_{50} and MIC_{90} results of all isolates, as well as the number and percentage of susceptible isolates. Figure 1 shows the MIC distribution of the isolates towards streptomycin, enrofloxacin, novobiocin and trimethoprim/ sulfamethoxazole. Among 67 isolates tested, 97% were susceptible to enrofloxacin ($MIC_{50,}$ 0.12 µg/ml, MIC_{90} 2 µg/ml). Novobiocin ($MIC_{90,}$ 4 µg/ml) and streptomycin (MIC_{90}, 128 µg/ml) inhibited 85% of the isolates at the susceptible breakpoints. Moderate susceptibility (60 - 80%) was observed to trimethoprim/ sulfamethoxazole 77.6%, neomycin and florfenicol both at 62.7% of overall isolates. Extent of susceptibility was low (less than 50 - 60%) for tylosin tartrate (32.8%), clindamycin and sulphathiazole both at (23.9%). The susceptibility levels were very low (less than 10%) to ceftiofur (6%), spectinomycin (6%) and tetracyclines/ oxtetracyclines (4.5%). In contrast, 0% overall susceptibility was exihibited for β-Lactams (amoxicillin, penicillin), gentamycin and erythromycin against Flavobacteriaceae isolates at the given susceptible breakpoints (Table 1). MIC values of control strains were within the acceptable range (CLSI, 2014)(Figure 1).

Antimicrobial susceptibility of Flavobacteriaceae isolates according to the sampling groups

The in vitro activity of the antimicrobial agents was also evaluated based on farmed and wild fish and sampling regions. Generally, susceptibility levels among isolates did not vary significantly between neither farmed versus

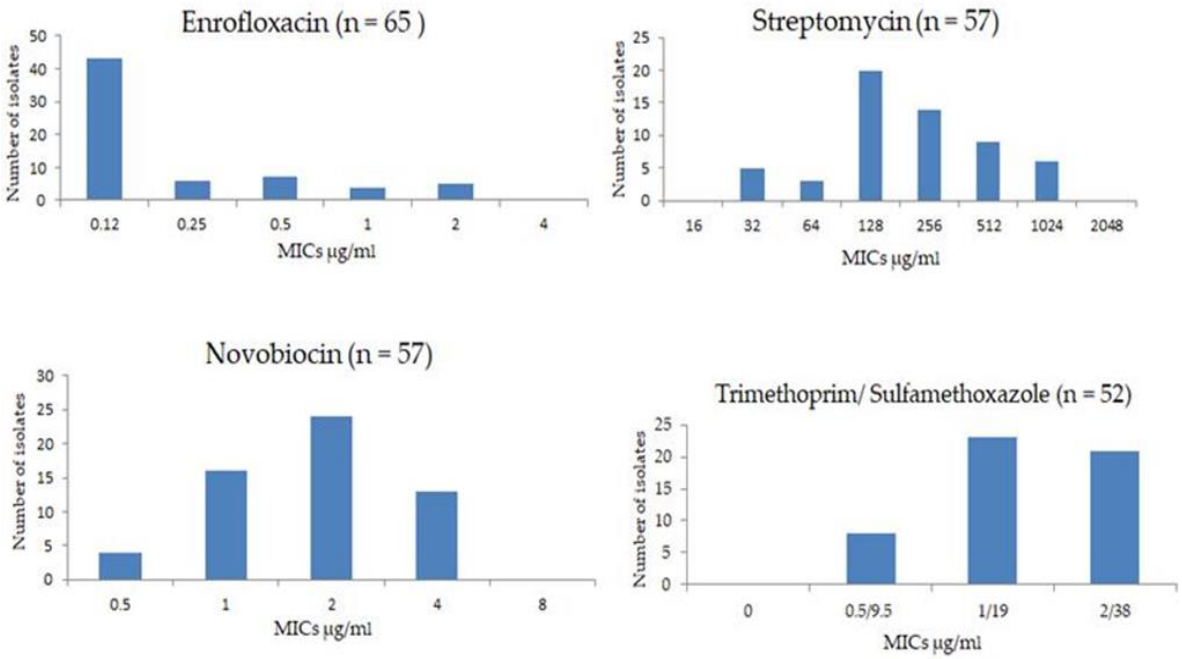

Figure 1. Distributions of Minimum Inhibitory Concentrations (MICs) values of the antimicrobial agent's streptomycin, enrofloxacin, novobiocin, trimethoprim and sulfamethoxazole for susceptible Flavobacteriaceae isolates.

wild fish nor sampling region. One observation was, however, that while none of the isolates from wild fish were susceptible to ceftiofur and tetracycline/ oxytetracycline, respectively three and four isolates from farmed fish were susceptible to these antimicrobials (Table 2).

Multiple antimicrobial resistance (MAR) index

Overall antimicrobial susceptibility pattern of each isolate is also summarized in Table 3. The susceptibility patterns varied within groups of isolates from the same site or sample, and also within the same species. Most of the *C. indologenes* isolates showed low susceptibility up to 17 of the 19 antimicrobials tested, which was the highest degree of multi-resistance observed in this study regardless of sample origin. The lowest level of resistance was observed in *F. indicum*, which was sensitive to 6 of 19 antimicrobials tested. Moreover, *C. indologenes* and *C. joostei*, which were isolated from almost all the sampling sites/fish, showed a broader range of MAR indexes. The highest MAR index was 0.89 observed in *C. indologenes* and the lowest was 0.32 observed in *C. indicum* isolates, indicating that none of the sampling sites could be characterized as low-risk sources of contamination. Comparison of the different culture system showed that all isolates recovered from wild and farmed Nile tilapia revealed multiple resistance to all antimicrobials tested.

DISCUSSION

Antimicrobial resistance has become a global health threat involving the environmental, animal and human sectors. Knowledge of antimicrobial susceptibility and resistance patterns in microbes from many biological compartments is important to understand the development and transmission of resistance within and between bacterial reservoirs as well as identifying treatment alternatives against specific infections. In this study, we report data on antimicrobial susceptibility of Flavobacteriaceae isolated from wild and farmed Nile tilapia in Tanzania. Our results show variable antimicrobial susceptibility patterns against 19 antimicrobial agents commonly used in veterinary medicine and aquaculture in most developed countries.

This is in concordance with previous studies showing that Flavobacteriaceae isolates are able to grow in high concentrations of several antimicrobial agents, suggesting the presence of intrinsic or acquired resistance mechanisms, resulting in reduced susceptibility to multiple antimicrobial agents (Akinbowale et al., 2006, Clark et al., 2009, Henríquez-Núñez et al., 2012, Hesami et al., 2010).

All isolates were resistant to the β-lactams amoxicillin and penicillin, to the aminoglycoside gentamycin, the macrolide erythromycin and to sulfadimethoxine. Resistance to β-lactams was expected as the commonly chromosomally encoded Amber Class B and other β-lactamase enzymes have been previously broadly recognized in Flavobacteriaceae (Bellais et al., 2002b,

Table 2. In vitro activities of selected antimicrobials against Flavobacteriaceae isolates according to the culture system.

Classes	Antimicrobial agent	Farmed fish isolates (N=44)	Wild fish isolates (N=23)
		Susceptible isolates n (%)	Susceptible isolates n (%)
β-Lactams	Amoxicillin	0 (0)	0 (0)
	Penicillin	0 (0)	0 (0)
	Ceftiofur	4 (9.1)	0 (0)
Aminoglycosides	Gentamicin	0 (0)	0 (0)
	Spectinomycin	3 (6.8)	1 (4.3)
	Streptomycin	37 (84.1)	20 (87)
	Neomycin	29 (65.9)	13 (56.5)
Sulfonamides	SXT	33 (75)	19 (82.3)
	Sulphathiazole	13 (29.5)	3 (13)
	Sulphadimethoxine	0 (0)	0 (0)
Tetracyclines	Tetracycline	3 (6.8)	0 (0)
	Oxytetracycline	3 (6.8)	0 (0)
Macrolides	Tylosin tartrate	19 (43.2)	3 (13)
	Erythromycin	0 (0)	0 (0)
Fluoroquinolones	Enrofloxacin	43 (97.7)	22 (95.7)
Phenicols	Florfenicol	32 (73)	13 (56.5)
Coumarins	Novobiocin	38 (86.4)	19 (82.6)
Lincomycins	Clindamycin	13 (29.5)	3 (13)

Bellais et al., 2002a; Gonzalez and Vila, 2012). Resistance to erythromycin is also consistent with previous studies, in which MICs of > 32 µg/ml have been reported amongst Flavobacteriaceae (Clark et al., 2009, Darwish et al, 2008, Declercq et al., 2013). In contrast to the present investigation, a previous study reported 100 % susceptibility (MIC90 >8µg/ml) to gentamycin in *Flavobacterium columnare* isolates collected worldwide from 17 fish species (Declercq et al., 2013).

Most (97%) Flavobacteriaceae isolates were susceptible to fluoroquinolone enrofloxacin. The variable levels of susceptibility found for streptomycin, novobiocin and sulfamethoxazole/ trimethoprim tested in this study, suggest that they may be useful for treatment of infections caused by some strains. However, sensitivity testing should be performed against the actual disease strain before any of these antimicrobials are considered for use. Variation in antimicrobial susceptibility amongst Flavobacteriaceae and other fish bacteria have been reported elsewhere (Akinbowale et al., 2006, Schmidt et al., 2000). In general, Flavobacteriaceae are recognized as intrinsically resistant to several antimicrobial agents, and only a few specific transferable resistance determinants known from other bacterial genera have been identified. Several genes coding for efflux pumps have been associated with intrinsic resistance in Flavobacteriaceae isolates. For example, previous study investigated genome sequence of *Flavobacterium johnsoniae* and revealed presence of chloramphenicol inducible multidrug efflux pump system of RND family (Clark et al., 2009, Henríquez-Núñez, 2012, Michel et al., 2005). In addition, the low susceptibility of Flavobacteriaceae to florfenicol has been associated with expression of multidrug efflux pumps (Michel et al., 2005). Moreover, it has been demonstrated that efflux pumps are important for other processes such as detoxification of intracellular metabolites, bacterial virulence, cell homeostasis and intercellular signal trafficking (Martinez et al., 2009; Pasqua et al., 2019).

Most isolates in the present study were inhibited by low concentrations of florfenicol, but since 37% were able to grow in higher florfenicol concentrations, the presence of acquired resistance mechanisms cannot be ruled out. The banning of use of phenicol, due to its adverse effect in humans, has probably aided in reducing the risk of acquired resistance in aquaculture environments (Akinbowale et al., 2006; Tsai et al., 2019). However, other studies have insisted that monitoring resistance of florfenicol is of great importance as it is the main antimicrobial agent used to treat infections caused by

Table 3. MAR index of the Flavobacteriaceae isolates (N = 67).

Isolates	Species	Culture system	Year of isolation	Number of antibiotics (N = 19) to which isolate was resistant	MAR index
MZ9L	*C. joostei*	Wild	Nov 2015	11	0.57
MZ81GL	*C. joostei*	Wild	Nov 2015	12	0.63
MZ84GL	*C. joostei*	Wild	Nov 2015	11	0.57
MZ18L	*C. joostei*	Wild	Nov 2015	10	0.53
MZ32L	*C. joostei*	Wild	Nov 2015	13	0.68
MZ26GL	*C. joostei*	Wild	Nov 2015	15	0.78
MZ3L	*C. joostei*	Wild	Nov 2015	14	0.74
MZ20KDS	*C. indologenes*	Wild	Nov 2015	12	0.63
MZ60L	*C. indologenes*	Wild	Nov 2015	15	0.79
MZ4GL	*C. indologenes*	Wild	Nov 2015	12	0.63
MZ12GL	*C. indologenes*	Wild	Nov 2015	15	0.79
MZ13GL	*C. indologenes*	Wild	Nov 2015	15	0.79
MZ52GL	*C. indologenes*	Wild	Nov 2015	15	0.79
MZ50GL	*C. indologenes*	Wild	Nov 2015	11	0.58
MZ83GL	*C. indologenes*	Wild	Nov 2015	10	0.53
MZ60KDS	*C. indologenes*	Wild	Nov 2015	13	0.68
MZ31GL	*C. indologenes*	Wild	Nov 2015	13	0.68
MZ119L	*C. indologenes*	Wild	Nov 2015	14	0.74
MZ32GL	*C. indologenes*	Wild	Nov 2015	13	0.68
MK8GL	*C. indologenes*	Wild	Nov 2015	14	0.74
MZ14GL	*C. species*	Wild	Nov 2015	12	0.63
MZ2GL	*C. species*	Wild	Nov 2015	14	0.74
MZ10L	*C. species*	Wild	Nov 2015	14	0.74
MGL22L	*F. aquatile*	Farmed	Apr 2016	10	0.53
TNM16GL	*F. aquatile*	Farmed	May 2016	10	0.53
DKM2GL	*F. aquatile*	Farmed	Febr 2016	12	0.63
DKM13GL	*C. species*	Farmed	Febr 2016	11	0.58
TNM37KDS	*F. oryzae*	Farmed	May 2016	11	0.57
MGL48GL	*F. oryzae*	Farmed	Apr 2016	10	0.53
DKM17KDS	*F. indicum*	Farmed	Febr 2016	6	0.32
MGL37KDS	*F. indicum*	Farmed	Apr 2016	9	0.47
TNM8GL	*F. indicum*	Farmed	May 2016	11	0.57
MK27KDS	*C. joostei*	Farmed	Febr 2016	8	0.42
MK49GL	*C. joostei*	Farmed	Febr 2016	9	0.47
MGL50L	*C. joostei*	Farmed	Apr 2016	8	0.42
DKM11L	*C. joostei*	Farmed	Febr 2016	9	0.47
MK45GL	*C. joostei*	Farmed	Febr 2016	11	0.58
DKM8GL	*C. joostei*	Farmed	Febr 2016	12	0.63
DKM3GL	*C. joostei*	Farmed	Febr 2016	9	0.47
MGL28GL	*C. joostei*	Farmed	Apr 2016	13	0.68
MGL53L	*C. joostei*	Farmed	Apr 2016	12	0.63
MK40GL	*C. indologenes*	Farmed	Febr 2016	13	0.68
TNM34KDS	*C. indologenes*	Farmed	May 2016	17	0.89
MGL24GL	*C. indologenes*	Farmed	Apr 2016	17	0.89
MGL31GL	*C. indologenes*	Farmed	Apr 2016	15	0.79
DKM5L	*C. indologenes*	Farmed	Febr 2016	12	0.63
MGL54GL	*C. indologenes*	Farmed	Apr 2016	11	0.58
TNM13KDS	*C. indologenes*	Farmed	May 2016	15	0.79
MK45L	*C. indologenes*	Farmed	Febr 2016	12	0.63
MK5GL	*C. indologenes*	Farmed	Febr 2016	13	0.68

Table 3. Contd.

DKM12L	***C. indologenes***	**Farmed**	**Febr 2016**	**12**	**0.63**
DKM8L	*C. indologenes*	Farmed	Febr 2016	10	0.52
DKM11GL	*C. indologenes*	Farmed	Febr 2016	16	0.84
MGL22L	*C. indologenes*	Farmed	Apr 2016	16	0.84
TNM21GL	*C. species*	Farmed	May 2016	15	0.79
TNM34L	*C. species*	Farmed	May 2016	12	0.63
MGL8GL	*C. species*	Farmed	Apr 2016	11	0.58
TNM14GL	*C. species*	Farmed	May 2016	12	0.63
MGL57GL	*C. species*	Farmed	Apr 2016	12	0.63
MGL26L	*C. species*	Farmed	Apr 2016	12	0.63
TNM17L	*C. species*	Farmed	May 2016	12	0.63
TNM34L	*C. species*	Farmed	May 2016	13	0.68
TNM19GL	*C. species*	Farmed	May 2016	13	0.68
TNM40GL	*C. species*	Farmed	May 2016	13	0.68
TNM33L	*C. species*	Farmed	May 2016	13	0.68
TNM10GL	*C. species*	Farmed	May 2016	12	0.63
DKM13L	*C. species*	Farmed	Febr 2016	10	0.53

Note: isolate code MGL: Mgeta Langali, TNM: Tangeni, DKM: Dakawa, MK: Mkindo, MZ: Mwanza, GL: Gills, KDS: Kidney, L: Liver, C: Chryseobacterium and F: Flavobacterium.

Flavobacteriaceae (Verner-Jeffreys et al., 2015). Unfortunately, no information regarding the use of florfenicol or other phenicol compounds was available in the sampling areas.

Tetracycline and/or oxytetracycline have been frequently used in fish farming, particularly to control systemic bacterial infections of fish in most developed countries (Miranda et al., 2018; Jerbi et al., 2011), and it has been found that the continued and widespread use of tetracycline has led to the development of resistant bacteria in all aspects of fish farming (Higuera-Llantén et al., 2018; Mirand and Zemelman, 2002). This study revealed low susceptibility to tetracycline and/or oxytetracycline, with MIC ranges of 0.25 - 8 µg/ml. Other workers recorded considerable frequencies of tetracycline resistance amongst Flavobacteriaceae isolated from aquaculture environments and associated it with imprudent use of antimicrobials and acquired resistance (Schmidt et al., 2000). However, due to lack of information relating to the treatment history of the sampled fish, it is difficult to associate the low susceptibility observed with acquired resistance or imprudent use of antimicrobials.

All Flavobacteriaceae were found with MAR index values greater than 0.2 in this study. Previous studies have shown that bacteria originating from an environment where several antimicrobials are used usually display MAR indexes greater than 0.2 (Tambekar et al., 2006). Thus, the fact that all MAR indexes recorded were above 0.2 indicate that they are from the high risk source where antibiotics are frequently used, possibly from surrounding study areas. Furthermore, low susceptibility results in this study concur with the findings of previous studies (Clark et al., 2009), who found multidrug resistance in *Flavobacteria* and *Chryseobacterium* species isolated from fish. Nile tilapia is the most cultured and widely consumed fish species in Tanzania. Lake Victoria is the major capture fish source in the country and also is a source for tilapia fingerlings to most fish farmers in Tanzania. The assessment of Antimicrobial resistance (AMR) is of great importance as dissemination of AMR bacteria to fish could lead to serious public health risks. Interviews conducted in our earlier study did not reveal a history of antimicrobial use in farmed fish during the present study (Mwega et al., 2018). Our results suggest that isolates from wild fish were slightly more resistant against the antimicrobials than the isolates from farmed fish. Although the difference is not significant among groups, this observation could be explained by the fact that the wild Nile tilapia was captured at the shorelines of Lake Victoria which makes the two environments more alike. Systematic data on use of antimicrobials to the ponds are not available in Tanzania, so we do not know how much and how frequent antimicrobials are used. Therefore, it is not possible to draw any conclusions on the association between antimicrobials use and the levels of resistance observed in the present study.

Conclusion

In this study, we have found that Flavobacteriaceae isolates are resistant to several antimicrobials tested. The ability of Flavobacteriaceae to resist multiple drugs is indeed was expected, as it is known that most of these isolates are intrinsically resistant to several classes of antimicrobials and/ or can acquire resistant genes from

other environmental microbes. However, whether the antimicrobial resistance traits are acquired through gene transfer or intrinsic is not clearly elucidated at the moment. This can be determined by analyzing whole genomes of those isolates in the future. The findings from this study give us insight into appropriate choice of antimicrobial agents for effective treatment of infections caused by these isolates.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Akinbowale OL, Peng H, Barton MD (2006). Antimicrobial resistance in bacteria isolated from aquaculture sources in Australia. Journal of Applied Microbiology 100(5):1103-1113.

Bellais S, Naas T, Nordmann P (2002a). Molecular and biochemical characterization of Ambler class A extended-spectrum β-lactamase CGA-1 from Chryseobacterium gleum. Antimicrobial Agents Chemotherapy 46(4):966-970.

Bellais S, Naas T, Nordmann P (2002b). Genetic and biochemical characterization of CGB-1, an Ambler class B carbapenemhydrolyzing β-lactamase from Chryseobacterium gleum. Antimicrobial Agents Chemotherapy 46(9):2791-2796.

Bernardet JF, Bowman JP (2011). Genus I. Flavobacterium Bergey et al. 1923. In Bergey's Manual of Systematic Bacteriology, 2nd edn, vol. 4, pp. 112–154. Edited by W. Whitman. Baltimore: The Williams & Wilkins Co., Baltimore

Bernardet JF, Nakagawa Y (2006). An introduction to the family Flavobacteriaceae. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E, editors. The prokaryotes: Delta, Epsilon Subclass. Springer New York7:455-480.

Cabello FC (2006). Heavy use of prophylactic antibiotics in aquaculture, a growing problem for human and animal health and for the environment. Environmental Microbiology 8(7):1137-1144.

Cabello FC, Godfrey HP, Tomova A, Ivanova L, Dölz H, Millanao A (2013). Antimicrobial use in aquaculture re-examined, its relevance to antimicrobial resistance and to animal and human health. Environmental Microbiology 15(7):1917-1942.

Clark SE, Jude BA, Danner GR, Fekete FA (2009). Identification of a multidrug efflux pump in Flavobacterium johnsoniae. Veterinary Research 40(6):1-10.

Clinical and Laboratory Standards Institute (CLSI) (2014). Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated from Aquatic Animals, Approved Guideline VET04-A2. Clinical and Laboratory Standards Institute. Wayne, PA.

Darwish AM, Farmer BD, Hawke JP (2008). Improved method for determining antibiotic susceptibility of Flavobacterium columnare isolates by broth microdilution. Journal of Aquatic Animal Health: 20(4):185-191.

Darwish AM, Ismaiel AA, Newton JC, Tang J (2004). Identification of Flavobacterium columnare by a species-specific polymerase chain reaction and renaming of ATCC43622 strain to Flavobacterium johnsoniae. Molecular and cellular probes 18(6):421-427.

Declercq AM, Boyen F, Van Den Broeck W, Bossier P, Karsi A, Haesebrouck F, Decostere A (2013). Antimicrobial susceptibility pattern of *Flavobacterium columnare* isolates collected worldwide from 17 fish species. Journal of Fish Diseases 36(1):45-55 .

Gonzalez LJ, Vila AJ (2012). Carbapenem resistance in mediated by metallo-β-lactamase BlaB. Antimicrobial Agents Chemotherapy 56(4):1686-1692.

Gufe C, Canaan Hodobo T, Mbonjani B, Majonga O, Marumure J, Musari S, Machakwa J (2019). Antimicrobial Profiling of Bacteria Isolated from Fish Sold at Informal Market in Mufakose, Zimbabwe. International Journal of Microbiology.

Henríquez-Núñez H, Evrard O, Kronvall G (2012). Antimicrobial susceptibility and plasmid profiles of *Flavobacterium psychrophilum* strains isolated in Chile. Aquaculture 354:38-44.

Hesami S, Parkman J, Macinnes JI (2010). Antimicrobial susceptibility of *Flavobacterium psychrophilum* isolates from Ontario. Journal of Aquatic Animal Health 22(1):39-49.

Higuera-Llantén S, Vásquez-Ponce F, Barrientos-Espinoza B, Mardones FO, Marshall SH, Olivares-Pacheco J (2018). Extended antibiotic treatment in salmon farms select multiresistant gut bacteria with a high prevalence of antibiotic resistance genes. PLoS ONE 13(19).

Izumi S, Aranishi F (2004). Relationship between gyrA mutations and quinolone resistance in Flavobacterium psychrophilum isolates. Applied Environmental Microbiology 70(7):3968-3972.

Jensen LB, Angulo FJ, Molbak K, Wegener HC (2008). Human health risks associated with antimicrobial use in animals. Guide to Antimicrobial Use in Animals pp. 13-26.

Jerbi MA, Ouanes Z, Besbes R, Achour L, Kacem A (2011). Single and combined genotoxic and cytotoxic effects of two xenobiotics widely used in intensive aquaculture. Mutation Research 724(1-2):22-27.

Kumru S, Tekedar HC, Gulsoy N, Waldbieser GC, Lawrence ML, Karsi A (2017). Comparative Analysis of the Flavobacterium columnare Genomovar I and II Genomes. Frontiers in Microbiology 8:1375.

Loch TP, Faisal M (2015). Emerging flavobacterial infections in fish: A review. Journal of Advanced Research 6(3):283-300.

Loch TP, Fujimoto M, Woodiga SA, Walker ED, Marsh TL, Faisal M (2013). Diversity of fish-associated Flavobacteria of Michigan. Journal of Aquatic Animal Health 25(3):149-64

Madsen L, Dalsgaard I (2000). Comparative studies of Danish Flavobacterium psychrophilum isolates: ribotypes, plasmid profiles, serotypes, and virulence. Journal of Fish Diseases 23(3):211-218.

Martinez JL, S'anchez MB, Mart´ınez-Solano L (2009). Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. FEMS Microbiology Review 33(2):430-449.

McBride MJ (2014). The Family Flavobacteriaceae. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F. (eds) The Prokaryotes. Springer, Berlin, Heidelberg.

Michel C, Matte-Tailliez O, Kerouault B, Bernardet JF (2005). Resistance pattern and assessment of phenicol agents' minimum inhibitory concentration in multiple drug resistant Chryseobacterium isolates from fish and aquatic habitats. Journal of applied microbiology 99(2):323-332.

Mirand CD, Zemelman R (2002). Antimicrobial multiresistance in bacteria isolated from freshwater Chilean salmon farms. The Science of the total environment: 293(1-3):207-218.

Miranda CD, Godoy FA, Lee MR (2018). Current Status of the Use of Antibiotics and the Antimicrobial Resistance in the Chilean Salmon Farms. Frontiers in microbiology 9:1284.

Mwega E, Colquhoun D, Tuntufye H, Mdegela R, Mutoloki S, Evensen Ø, Wasteson Y (2018). Isolation and Characterization of Flavobacteriaceae from Farmed and Wild Nile Tilapia in Tanzania. Journal of Aquatic Animal Health 31(1):23-30.

Pasqua M, Grossi M, Zennaro A, Fanelli G, Micheli G, Barras F,

Colonna B, Prosseda G (2019). The Varied Role of Efflux Pumps of the MFS Family in the Interplay of Bacteria with Animal and Plant Cells. Microorganisms 7(9):285.
Pathmalal M (2018). Heavy use of antibiotics in aquaculture: Emerging human and animal health problems – A review. Sri Lanka Journal of Aquatic Science 23:13-27.
Pilarski F, Rossini AJ, Ceccarelli PS (2008). Isolation and characterization of Flavobacterium columnare (Bernardet et al. 2002) from four tropical fish species in Brazil. Brazillian Journal of Biology 68(2):409-414.
Ratnamani M, Rao R (2013). Elizabethkingia meningoseptica: Emerging nosocomial pathogen in bedside hemodialysis patients. Indian Journal of Critical Care Medicine 17(5):304-307.
Samuel L, Marian MM, Apun K, Lesley MB, Son R (2011). Characterization of Escherichia coli isolated from cultured catfish by antibiotic resistance and RAPD analysis. International Food Research 18(3):971-976.
Schmidt AS, Bruun MS, Dalsgaard I, Pedersone K, Larsen, JL (2000). Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. Applied Environmental Microbiology 66(11):4908-4915.
Tambekar DH, Dhanorkar DV, Gulhane SR, Khandelwal VK, Dudhane MN (2006). Antibacterial susceptibility of some urinary tract pathogens to commonly used antibiotics. African Journal of Biotechnology 5(17):1562-1565.
Tsai MY, Lin CF, Yang WC, Lin CT, Hung KH, Chang GR (2019). Health risk assessment of banned veterinary drugs and quinolone residues in shrimp through liquid chromatography–tandem mass spectrometry. Applied Sciences 9(12):2463.
Verner-Jeffreys D, Taylor NGH, Pitlochry, Perthshire (2015). Review of Freshwater Treatments Used in the Scottish Freshwater Rainbow Trout Aquaculture Industry. Scottish Aquaculture Research Forum Report SARF100 Available @: http://www.sarf. org.uk/cms-assets/documents/208213-793666.sarf100
Watts JEM, Schreier HJ, Lanska L, Hale MS (2017). The Rising Tide of Antimicrobial Resistance in Aquaculture: Sources, Sinks and Solutions. Marine Drugs 15(6):158.

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