East African Medical Journal Vol. 80 No. 4 April 2003
ARCOBACTER BUTZLIERI STRAINS FROM POULTRY ABATTOIR EFFLUENT IN NIGERIA
K.O. Amisu, BSc, MSc, A.O. Coker, MD, PhD, Department of Medical Microbiology and Parasitology, College of Medicine, University of Lagos, Idi-Araba, Lagos. Nigeria, S.L.W. On, PhD, Department of Bacteriology, Danish Veterinary Institute, Bulowsvej 27, DK- 1790 Copenhagen V, Denmark, R.D. Isokpehi, PhD, Department of Medical Microbiology and Parasitology, College of Medicine, University of Lagos, Idi-Araba, Lagos, Nigeria. Present address: South African National Bioinformatics Institute, University of the Western Cape, Bellville 7535, South Africa

Request for reprints to: K.O.Amisu, Campylobacteria Research Laboratory, Department of Medical Microbiology and Parasitology, College of Medicine, University of Lagos, Idi-Araba, Lagos, Nigeria

ARCOBACTER BUTZLIERI STRAINS FROM POULTRY ABATTOIR EFFLUENT IN NIGERIA

K.O. AMISU, A.O. COKER, S.L.W. ON and R.D. ISOKPEHI

ABSTRACT

Objective: **To investigate the prevalence, species distribution and genetic diversity of zoonotic *Arcobacter* species.**
Design: **Prospective study.**
Setting**: Drainage system of a cosmopolitan chicken abattoir in Lagos, Nigeria.**
Methods : **One hundred and fifity drainage water samples were enriched in a minimal antibiotics-containing medium at room temperature and bacteria then isolated by use of a membrane filtration method.**
Results: **Twenty six (14%) of samples were positive for *Arcobacter spp.* Of these, 20 were examined by a comprehensive probabilistic identification scheme for *Epsilobacteria* and all strains identified as *A. butzleri.* AFLP analysis of these strains revealed considerable genetic diversity among the strains, with 12 genotypes defined at the 90% similarity level.**
Conclusion**: The prevalence *of A. butzleri* in Nigerian poultry abattoir effluent indicates this species may constitute a public health problem in this country. AFLP profiling could be a useful tool for molecular epidemiological and population genetic studies of this organism. This is the first known report of *A. butzleri* in Nigeria, and first application of AFLP analysis for genotyping the species.**

INTRODUCTION

Arcobacter species are metabolically inactive, curved or spiral-shaped bacteria initially classified as *Campylobacter* species (1), although their ability to grow in air at low temperature (30°C) after primary isolation in microaerobic conditions was a marked difference. Organisms with this phenotype were later designated into a separate genus by Vandamme *et al*, (2) based on evidence from immunotyping, SDS-PAGE protein profiling, and DNA-DNA- and DNA-rRNA hybridization experiments. Four species are presently recognised: *Arcobacter cryaerophilus* [formerly *Campylobacter cryaerophila;* (3)], *A. nitrofigilis* [formerly *C. nitrofigilis*; (4)], *A. butzleri* [formerly *C. butzleri*; (5)], and *A. skirrowii* (6). Of these species, only *A. nitrofigilis* has not been associated with human and/or animal disease.

The socio-economic importance of *Arcobacter* spp, initially stemmed from their frequent association with diseases of livestock. These include mastitis in milk producing cows, septic abortion in cattle and pigs, diarrhoea, and infertility problems in cattle (3,7-9). However, since re-classification(6), *Arcobacter* spp. have emerged from obscurity to be recognised as food borne pathogens of public health importance (10). Outbreaks and sporadic case reports of human *Arcobacter* infections are mainly those of gastroenteritis (5,11-13).

Arcobacter spp have been isolated from a wide range of food and water sources. They have been detected in poultry and animal carcasses (14,15), sewage, wells, rivers and drinking water (16-18). It has been suggested that *Arcobacter* gastroenteritis may be more prevalent in developing countries because of the poor level of hygiene and inadequate supply of good drinking water (19).

In this study, we investigated the presence of *Arcobacter* spp in drainage water samples from the washings of chicken carcasses in a Nigerian abattoir, and characterized strains by phenotypic and genotypic methods to determine their taxonomic and genomic diversity.

MATERIALS AND METHODS

Samples and location studied: Untreated wastewater effluents of a chicken abattoir in Lagos metropolis (Mushin) were used. Different birds were slaughtered in this abattoir and wastewaters from the washings of their carcasses discharged directly into a shallow open drainage that are linked to a major drainage system.

Collection and enrichment of samples: One hundred and fifty 5ml samples of wastewater effluent were collected at random over a period of four months (May-August 2000) and

transported within 30 min of collection to the laboratory. About 0.5 ml of slightly turbid supernatant of the test samples were added to 4.5 ml sterile brain-heart infusion broth (Oxoid Ltd., Basingstoke, UK) containing 0.5% yeast extract, *Campylobacter* selective antibiotic supplement (SR 155, Oxoid), giving final concentrations of 0.032 mg/ml cefoperazone and 0.01 mg/ml amphotericin), and 0.01 mg/ml pharmaceutical vancomycin hydrochloride (American Pharmaceutical Partners Inc., Los Angeles, USA) (BHI-CVA broth). The antibiotic formulation concurred with commercially produced cefoperazone-vancomycin-amphoterin (CVA) media that is used for isolation of *Arcobacter* spp. (20). Cultures were incubated for 18-20 hours at room temperature (28-29 ^{0}C) with the caps of the containers slightly loosened to select for aerobic growth at low temperature (21).

Isolation of Arcobacter strains from enrichment cultures: 1 ml aliquots of enrichment culture were diluted in sufficient sterile nutrient broth so as to produce a faintly turbid suspension and then inoculated by both direct and membrane filter techniques onto BHI-CVA agar media containing 5-7% v/v sheep blood. For membrane filter isolation, sterile 0.45µm pore sized cellulose acetate membrane filters (Advantec Ltd., Tokyo, Japan) were placed onto the media and 5-7 drops of diluate added onto the filter surface. Membrane filters were then removed aseptically after incubation for 30 min at room temperature. All inoculated plates were incubated in a candle extinction jar (22) for 24-48 hours at 37°C. Plates with poor growth but which did not show presence of *Proteus* or confluent growths of contaminants were usually incubated for an additional 24 hours. Bacterial colonies on primary isolation plates that resembled those of *Campylobacter* species were subcultured for further testing.

Identification of isolates: Isolates were presumptively classified as *Arcobacter spp.* by determination of, (i) cell morphology through examining gram-stained films (using strong carbol-fuchsin as counter stain) under light microscopy, (ii) oxidase and (iii) catalase activity, (iv) ability to ferment glucose and lactose and (v) growth in atmospheric air at room temperature (28-29 ^{0}C) by use of previously described methods, (14,23). Motility, and urease and indole production were determined by use of motility-indole urea medium (Bio-Life, Ljusne, Sweden) according to the manufacturers instructions. Gram-negative curved or spiral rod-shaped, motile, aerotolerant, asaccharolytic bacteria that grew on MacConkey agar and produced oxidase and catalase but not urease or indole were considered potential *Arcobacter* spp. Species identity of 20 of the isolates was determined by extensive (>60 traits) phenotypic characterisation and probabilistic comparison of data with similar results for 37 *Campylobacter, Arcobacter, Helicobacter* and related taxa, as described previously (24).

AFLP profiling: The genetic diversity of 20 strains was examined by Amplified Fragment Length Polymorphism (AFLP) analysis by use of the methods described previously for *Campylobacter* spp. (25). Digitized patterns were analysed by use of the program BioNumerics 2.5 (Applied Maths, Kortrijk, Belgium). Bands were defined by use of automated search parameters detecting fragments with a minimal area of 1.5% of total pattern area, an intensity of 9.0% greater than the background fluorescence and a shoulder sensitivity of 5.0 to improve detection or hands with near-identical molecular sizes. Similarities between strain profiles were calculated using the Dice coefficient and strain relationships inferred by Unweighted Pair-Group Mathematical Average (UPGMA) clustering. Strains 8, 16, 18, 20, 41 and 45 were examined on two different occasions to determine reproducibility of the AFLP analysis.

RESULTS

An enrichment method in conjunction with membrane filtration was found suitable for isolation of *Arcobacter* species from wastewater effluent of chicken abattoir. Twenty six (14%) of the samples examined were positive for *Arcobacter* strains based on presumptive identification by the salient phenotypic criteria described above. Twenty strains were chosen at random for extensive phenotypic characterisation, of which, 19 had identification (ID) scores (Willcox probability percentage values) to *A. butzleri* exceeding 98% while one isolate (Lagos 26) attained an ID score of 83% to this species.

Figure 1

AFLP band patterns of 20 Nigerian Arcobacter butzleri strains. The scale bar denotes percentage similarity between patterns based on the Dice coefficient and UPGMA clustering

AFLP assay recognised 12 genotypes (among 20 *A. butzleri* strains) at the 90% similarity level (Figure 1). Furthermore the reproducibiilty of the assay using six strains was 89.95% (±SD 1.48).

DISCUSSION

Initial attempts to isolate *arcobacters* via direct plating onto selective media (BHI-CVA and modified *Butzleri* Type Medium; (22) commonly used in Nigeria to isolate the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* (26) frequently resulted in confluent growth of other bacterial and/or fungal species within 18 hours of incubation. Consequently this method was discontinued. Similar results have been reported elsewhere when examining porcine abortion material for *arcobacters* (9) and these data indicate that present direct plating methods are not optimised for *Arcobacter* isolation where microbiologically complex samples are examined, and/or where levels of *Arcobacter* contamination are relatively

low. By contrast, use of an enrichment method in conjunction with membrane filtration resulted in the recovery of 26 strains (representing 14% of the samples examined) that were presumptively classified as *Arcobacter spp.* by salient phenotypic criteria as described above. Our results further illustrate the suitability of membrane filtration for isolation of *Arcobacter* spp, and other campylobacteria with fastidious growth requirements and/ or marked susceptibility to antibiotics used in selective media (17,18,20,27,28).

Accurate identification of *Arcobacters* and related *Epsilobacteria* (including *Campylobacter., Helicobacter* and other organisms: (29) is well known to be problematic (24). This factor may have contributed to the limited information concerning the natural habitat, incidence and modes of transmission of *Arcobacter* species to man and animals (14). In this study, each of the 20 strains chosen at random for extensive phenotypic characterisation were confidently identified as *A. butzleri* by the scheme of On *et al*. (30), with identification (ID) scores (Willcox probability percentage values) to this species exceeding 98% for 19/20 strains. One isolate (Lagos 26) attained an ID score of 83%, to *A. butzleri,* a result due to atypical (all negative) results in triphenyl-tetrazolium chloride, potassium permanganate and nalidixic acid tests. Nonetheless the data illustrate the efficacy of this approach for identifying *Epsilobacteria,* as demonstrated in previous studies (14,31).

The predominance of *A. butzleri* in the poultry abattoir effluent in this study resembles that seen in studies of poultry and poultry products, where this species is significantly more common than other *Arcobacter* spp. (14,15,21,32). Similarly, *A. butzleri* is the sole or predominant *Arcobacter* species recovered from drinking and river water (17,33,34), indicating effective adaptation to survival and dissemination in an aqueous environment; observations with epidemiological implications to both animals and humans. Although *A. Butzleri* appears sensitive to disinfectants used to treat sewage (35), transmission via contaminated water is clearly a risk where exposure to untreated water occurs. Such conditions are likely in animal housing facilities and in countries with poorly developed sanitation conditions *Arcobacters* are psychrophilic they tend to grow best at temperatures lower than 30 ^{0}C and can even grow at 15 ^{0}C. Although most strains grow at 37 ^{0}C, relatively few (25%) grow at 42 ^{0}C (30). Thus their observation in a tropical country such as Nigeria is actually a rather interesting extension of our knowledge of their ecology.

These data illustrate the public health risk potential of *A. butzleri* and it is noteworthy that this species was recently isolated from 24.6% of human diarrhoea cases using appropriate isolation methods (36).

Few studies have examined the genetic diversity of *A. butzleri.* Ribotyping of 64 unrelated strains by their epidemiology revealed 50 different patterns, of which strains from the USA examined appeared to be more conserved than those studied From Australia, Thailand and Northern Ireland (19). PCR fingerprinting using repetitive sequences allowed discrimination of outbreak strains from 10 unrelated isolates, although considerable similarity among the patterns was seen (37). The efficacy and sensitivity of AFLP profiling for genotyping is well established (38) and we believe our study is the first to describe the application of AFLP fingerprinting to A. *butzleri*. This method was first developed for *Campylobacter* spp. (25) and its efficacy for examining *A. butzleri* suggests it may be useful for characterizing other members of the *Campylobacteraceae* including *Sulfurospirillum* and *Bacteroides ureolyticus*. Reproducibility of the AFLP assay was determined as 89.95% (± SD 1.48) and its high discriminatory potential is confirmed by recognition of 12 genotypes (among 20 *A. butzleri* strains) at the 90% similarity level (Figure 1). Thus, AFLP is a promising method for epidemiological investigations of this species.

Many common fragments were observed in the AFLP profiles (Figure 1) an observation consistent with the strains belonging to a well-defined species and possibly indicating a clonal relationship among the strains. The latter is to some extent corroborated by previous studies, (19,37), but more data are required to better establish the level of genetic diversity and implications for population structure of A. *butzleri*. Further studies are underway.

It is possible that the prevalence of *Arcobacter* species reported here is an underestimate. The candle jar method used here is inexpensive and often used in developing countries but does not provide an optimal microaerobic environment (39). Furthermore, cultivation of certain *Arcobacter spp.* (notably *A. skirrowii)* is more difficult than others (14,32), Nevertheless our report is, to our knowledge, the first to confirm these bacterial pathogens in Nigeria and it thus complements some two decades of studies on *Campylobacter* in the country (40). The accumulation of data indicating the zoonotic and pathogenic potential of these organisms supports the need for further studies of their prevalence, distribution and significance in both developing and developed countries. Additional studies on the ecological diversity; and the public health importance of the organism in Nigeria are in progress.

ACKNOWLEDGEMENTS

To Penny Jordan and Kenn Kristiansen for excellent technical assistance.

REFERENCES

1. Neill, S.D., Ellis, W.A. and O'Brien, J.J. Designation of aerotolerant *Campylobacter* like organisms from porcine and bovine abortions due to the genus *Campylobacter. Res. Vet. Sci.* 1979; **27:**180-186.
2. Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R. and De Ley, J. Revision of *Campylobacter, Helicobacter,* and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int. J. Syst. Bacteriol.* 1991; **41:**88-103.

3. Neill, S.D., Campbell, J.N., O'Brien, J.J., Weatherup, S.T.C. and Ellis, W.A. Taxonomic position of *Campylobacter cryaerophila* sp. nov, *Int. J. Syst. Bacteriol.* 1985; **35:**342-356.
4. McClung, C.R., Patriquin, D.G. and Davis, R,E. *Campylobacter nitrofigilis* sp, nov., a new nitrogen-fixing bacterium associated with roots of *Spartina alterniflora* Loisel.*Int. J. Sist. Bacteriol.* 1983; **33:**605-612.
5. KiehIbauch, J.A., Brenner, D.J., Nicholson. *et al. Campylobacter butzleri* sp. nov. isolated from humans and animals with diarheal illness. *J. Clin. Microbiol*. 1991; **29:**376-385.
6. Vandamme, P., Vancanneyt, M., Pot, B., *et al.* Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter butzleri* comb. nov. and *Arcobacter skirrowii* sp. nov., an aerotolerant bacterium isolated from veterinary specimens. *Int. J. Syst. Bacteriol.* 1992; **42:**344-356.
7. Ellis, W.A., Neill, S.D., O'Brien, J.J., Ferguson, H.W. and Hanna, J. Isolation of *Spirillum/Vibrio*-like organisms from bovine fetuses. *Vet. Rec.* 1977; **100:**451-452.
8. Logan, E.F., Neill, S.D. and Mackie, D.P. Mastitis in diary cows associated with an aerotolerant *Campylobacter*. *Vet. Rec.* 1982; **110:**229-230.
9. On, S.L.W., Jensen, T.K., Bille-Hansen, V., Jorsal, S.E. and Vandamme, P. Prevalence and diversity of *Arcobacter* spp. isolated from the internal organs of spontaneous porcine abortions in Denmark. *Vet. Microbiol.* 2002; **85:**159-167.
10. Meng, J. and Doyle, M.P. Emerging issues in microbiological food safety. *Ann. Rev. Nutr.* 1997; **17:**255-275.
11. Taylor, D.N., Kiehlbauch, J.A., Tee, W. L., Pitarangsi, C. and Echeverria, P. Isolation of group 2 and aerotolerant *Campylobacter* species from Thai children with diarrhea. *J. Infect. Dis.* 1991; **163:**1062-1067.
12. Vandamme, P., Pugina, P., Benzi, G., *et al.* Outbreak of recurrent abdominal cramps associated with *Arcobacter butzleri* in an Italian school. *J Clin. Microbiol.* 1992; **30:** 2335-2337.
13. Lerner, J., Brumberger, V. and Preac-Mursic, V. Severe diarrhea associated with *Arcobacter butzleri Eur. J Clin. Microbiol. Inject. Dis.* 1994; **13:**660-662.
14. Atabay, H.l., Corry, J.E.L. and On, S.L.M. Diversity and prevalence of *Arcobacter* spp. in broiler chickens. *J. Appl. Microbiol.* 1998; **84:**1007-1016.
15. Manke, T. R., Wesley, I.V., Dickson, I.S. and Harmon, K.M. Prevalence and genetic variability of *Arcobacter* species in mechanically separated turkey. *J. Food Prot.* 1998.;**61:**1623-1628.
16. Jacob, J., Lior, H. and Feuerpfeil, I. Isolation of *Arcobacter butzleri* from a drinking water reservoir in Eastern Germany. *Zentralbl. Bakteriol.* 1993; **193:**557-562.
17. Stampi, S., Varoli, O., Zanetti, F. and De Luca, *G. Arcobacter cryaerophilus* and thermophilic campylobacters in a sewage treatment plant in Italy: two secondary treatments compared. *Epidemiol. Infect.* 1993; **110:**633-639.
18. Rice, E.W., Rodgers, M.R., Wesley, I.V., Johnson, C.H. and Tanner, S.A. Isolation of *Arcobacter butzleri* from ground water. *Lett. Appl. Microbiol.* 1999; **28:**31-35.
19. Kiehlbauch, J.A., Brenner, Plikaytis, B.D., Swaminathan, B., Cameron, D.N. and Wachsmuth, I.K. Restriction fragment length polymorphisms in the ribosomal genes for species identification and subtyping of aerotolerant *Campylobacter* species. *J. Clin. Microbiol.* 1991; **29:**1670-1676.
20. Anderson, K.F., Kielfibauch, J.A., Anderson, D.C., McClure, H.M. and Wachsmuth, I.K. *Arcobacter*(*Campylobacter*) *butzleri*-associated diarrheal illness in a nonhuman primate. *Infect. Immun.* 1993; **61:**2220-2223.
21. Lammerding, A.M., Harris, J.E., Lior, H., Woodward, D.E, Cole, L. and Muckle, C.A. Isolation method for recovery of *Arcobacler butzleri* from fresh poultry and poultry products. Newell, D.G., Ketley, J.M. and Feldman, R.A. (Eds) *Campylobacter, Helicobacters and Related Organisms* pp. 329-333. Plenum Press, New York. 1996.
22. Coker, A.O. and Dosunmu-Ogunbi, O. Isolation of *Campylobacter jejuni* Lagos- Nigeria a new bacterial agent of diarrhoea. *East. Afr. Med J* .1984; **61:**52-55.
23. Nachamkin.I *Campylobacter* and *Arcobacter.* Murray, P.R., Baron, E.J.O., Pfaller, M.A., Tenover, F.C., Yolken, R.H. (Eds) *Manual of Clinical Microbiology, sixth edition* pp. 483-491. ASM Press, Washington, 1995.
24. On, S.L.W. Identification methods for *campylobacters, helicobacters* and related organisms. *Clin. Microbiol. Rev.* 1996; **9:**405-422.
25. On, S.L.W. and Harrington, C.S. Identification of taxonomic and epidemiological relationships among *Campylobacter* species by numerical analysis of AFLP profiles. *FEMS Microbiol. Lett.* 2000; **193:**161-169.
26. Coker, A.O. and Obi, C.L. A comparison of two selective media for the isolation of *Campylobacter jejuni* in Lagos Nigeria. *Discov. Innov.* 1995; **7:**57-59.
27. Goossens. H., De Boeck, M., Coignau, H., V1aes, L., Van den Borre, C. and Butzler, J.P. Modified selective medium for isolation of *Campylobacter* spp. from feces: comparison with Preston medium, a blood-free medium, and a filtration system. *J. Clin. Microbiol.* 1986; **24:**840-843.
28. Le Roux, E. and Lastovica, A.L The Cape Town protocol; how to isolate the most campylobacters for your dollar, pound, franc, yen, etc. Lastovica, A.J., Newell, D.G.and Lastovica, E.E. (Eds). *Campylobacter, Helicobacter and Related Organisms pp.* 30-33. University of Cape Town, Cape Town, 1998.
29. Cavalier-Smith, T. The neomuran origin of archaebacteria, the negibacterial root of the universal tree and bacterial megaclassification. *Int. J. Syst.Evol. Microbiol.* 2002; **52:** 7-76.
30. On, S.L.W., Holines, B. and Sackin, M.J. A probability matrix for the identification of campylobacters, helicobacters, and allied taxa. *J. Appl. Bacteriol.* 1996; **81:**425-432.
31. On, S.L.W., Alabay, H-I. and Harrington , C.S. Evaluation of a probability matrix for identifying campylobacteria. Lastovica, A.J., Newell, D.G. and Lastovica. E.E. (Eds).*CampyIobacter, Helicobacter and Related Organisms pp.* 232-235. University of Cape Town, Cape Town, 1998.
32. Houf, K., Devriese, L.A., De Zutter, L. Van Hoof, J. and Vandamme, P. Development of a new protocol for the isolation and quantification of *Arcobacter* species from poultry products. *Int. J . Food. Microbiol.* 2001; **71:**189-196.
33. Musmanno, R,A., Russi, M., Lior, H. and Figura, N.*In vitro,* virulence factors of *Arcobacter butzleri* strains isolated from superficial water samples. *New microbiol.* 1997; **20:**63-68.
34. Jacob J., Woodward, D., Feuerpfeil, I. and Johnson, W.M., Isolation of *Arcobacter butzleri* in raw water and drinking water treatment plants in Germany. *Zentralbl Hyg Umweltmed.* 1998; **201:**189-198.
35. Zanetti, R, Stampi, S. De Luca, G, Varoli, O. and Tonelli, E, Comparative disinfection of secondary-treated sewage with chlorine dioxide and bromine chloride. *Zentralbl. Hyg. Umweltmed.* 1996; **198:**567-579.

36. Vandenberg, O., Dedliste, A., VIaes, L, Ebraert, A,, Retore, P., Douat, N., Vardamme, P. and Butzler, J. Prevalence and clinical features of non *Jejuni/coli Campylobacter* species and related organisms in stool specimens. Abstracts, 11th International Work-shop on *Campylobacter, Helicobacter* and Related Organisms. *Inter. J . Med. Microbiol.* 2001; **291:**Suppl. 31, 144, abstract P-22.

37. Vandamme, P., Giesendorf, B.A.J., Van Belkum, A., *et al.* Discrimination of epidemic and sporadic isolates of *Arcobacter butzleri* by polymerase chain reaction-mediated DNA fingerprinting. J. *Clin. Microbiol.* 1993; **31:**3317-3319.

38. Savelkoul, P.H., Aarls, H.J., de Haas, J., *et al.* Amplified-fragment length polymorphism analysis: the state of an art. *J. Clin. Microbiol.* 1999; **37:**3083-3091.

39. Wang, W.L., Luechtefeld, N.W., Blaser, M.J. and Reller, L.B., Effect of incubation atmosphere and temperature on isolation of *Campyplobacter.jejuni* from human stools.*Can. J. Microbiol.* 1993; **29:**468-470.

40. Coker, A.O., Isokpehi, R.D. and Amisu, K. Campylobacteria research in Lagos, Nigeria. Mobley, H.L.T., Nachamkin, I and McGee, D., (Eds) *Abstracts of the 10th, International Workshop on Campytobacter, Helicobacter and Related Organisms- pp.* 24, abstract no. CD 12. University of Maryland, Baltimore. 1999.