Invited Review

Rickettsioses in Sri Lanka – A mini review

Veranja Liyanapathirana

Sri Lankan Journal of Infectious Diseases 2019 Vol.9 (1):1-12

DOI: http://dx.doi.org/10.4038/sljid.v9i1.8239

Abstract

Rickettsioses are a group of vector-borne diseases that have come to the limelight in Sri Lanka during the last two decades. Evidence for spotted fever group rickettsioses, scrub typhus and other related diseases have been reported from Sri Lanka in a geographically restricted manner. This review summarizes the work done locally, that are publicly accessible as of 24th November 2018 with keyword searches 'rickettsioses and Sri Lanka', and 'typhus and Sri Lanka', on PubMed and Google Scholar. There is a considerable body of literature on rickettsioses in Sri Lanka, particularly as a result of collaborations with international research groups. These indicate that rickettsioses are found throughout the country, in a geographically restricted manner.

Keywords: Rickettsioses, Typhus, Sri Lanka

Introduction

Rickettsial infections or rickettsioses are a group of vector-borne diseases. Organisms responsible belong to two genera; namely *Rickettsia* and *Orientia*. Other genera such as *Coxiella*, *Ehrlichia* and *Anaplasma* which were also originally classified among Rickettsial organisms are now in different taxonomic groups.[1] Rickettsioses were initially classified according to serological cross reactivity. However, they are also classified into different groups according to the causative organisms, vectors involved, clinical features and geographical distribution.[2,3,4]

[1]*Department of Microbiology, Faculty of Medicine, University of Peradeniya, Sri Lanka*

Address for correspondence: Dr Veranja Liyanapathirana, Department of Microbiology, Faculty of Medicine, University of Peradeniya Telephone: +94777060887 Email: veranjacl@yahoo.com, veranjacl@pdn.ac.lk

https://orcid.org/0000-0002-4356-2172

Received 28 January 2019 and accepted 14 March 2019

Typhus group

The typhus group consists of *Rickettsia prowazekii* which causes epidemic typhus and *Rickettsia typhi* (formerly known as *Rickettsia mooseri*) which causes endemic typhus. *Rickettsia prowazekii* is transmitted by *Pediculus humanus*, the human body louse. The organisms are excreted in the faeces of lice and humans acquire the organisms by inoculation when bite sites are scratched. This disease is typically associated with conditions of poor hygiene and overcrowding which promotes person to person spread of the body louse.[5] Rodents are the reservoirs of *Rickettsia typhi* which is transmitted by *Xenopsylla cheopis*, the rat flea via inoculation of faeces. Endemic typhus, also known as Murine typhus, is found in many parts of the world in urban areas with an abundance of rats.[5]

Spotted fever group (SFG)

Organisms belonging to this group are transmitted by ticks, except for *Rickettsia akari*, which is transmitted by mites and *Rickettsia felis* which is transmitted by fleas. The main vector for the transmission of spotted fever is ticks of the family *Ixodidae*. Tick – rickettsia interactions depend on the species of *Rickettsia*. Some *Rickettsia* species can be transmitted by only one species of ticks, whereas others may be transmitted by several tick species, either of the same genus or several different genera. How ticks acquire the organisms is still being debated, with the role of small mammals as reservoirs, co-feeding and sexual transmission being possible routes. However, transovarial passage of organisms clearly plays a role in the maintenance of rickettsiae within the tick population.[6]

Spotted fever group of rickettsiae include traditionally recognized pathogens, organisms recently recognized as being pathogenic and rickettsiae that have only been isolated from ticks. Prototype organisms of the spotted fever group are *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever and *Rickettsia conorii*, the causative agent of Mediterranean spotted fever. *Rickettsia japonica*, *Rickettsia africae*, *Rickettsia honei* and *Rickettsia slovaca* are some of the newer pathogenic rickettsiae belonging to the Spotted fever group.[6] The geographical distribution of spotted fever depends on the distribution of the vector.

Scrub typhus

Scrub typhus, also known as tsutsugamushi disease and chigger borne typhus, is caused by *Orientia tsustugamushi*. The larval or chigger stages of mites belonging to the genus *Leptotrombidium* are the primary vectors. In addition to being the vector, these mites also act as the natural reservoir for *Orientia tsutsugamushi* by maintaining the organism in nature transovarially and vertically. Scrub typhus is endemic in the Asia Pacific region and found associated with scrub jungles where grass of the Imperata family provides a niche for the mites.[7] *Orientia tsutsugamushi* has over 20 types of distinct antigenic strains, of which Gilliam, Kato and Karp were the initially characterized strains.[7] Recently, a novel *Orientia*

species, *Orientia chuto*, has been described from a patient in Dubai and from mites in Kenya, indicating the possibility of becoming an emerging pathogen.[8,9]

Other Rickettsioses

In addition to the above three groups of diseases, human ehrlichiosis and anaplasmosis, and Q fever caused by *Coxiella burnetii* are also frequently included under the term rickettsioses.

Rickettsioses in Sri Lanka

Earlier literature of rickettsioses in Sri Lanka

There are reports of outbreaks of scrub typhus among allied soldiers stationed in Ceylon during the world wars.[10,11] Two cases of murine typhus have been reported from Sri Lanka as far back as 1938 (Wijerama, 1930 and Fernando 1938 as quoted by Vasanthathilaka and Senanayake, 1995).[12] In a study conducted in 1972, the seropositivity of antibodies against scrub typhus detected by immunofluorescence assay (IFA) was found to be 6% among hospitalized patients, medical students and blood donors. This population was from the Western Province and the authors concluded that scrub typhus is unlikely to be of major public health concern.[13] Subsequently, a seroprevalence study among animals in Sri Lanka found 20% sero-positivity for *Rickettsia conorii* among goats and 30% sero-positivity among cattle.[14] Vasanthathilaka and Senanayake in 1995 reported a series of cases from Teaching Hospital, Peradeniya, where the diagnosis was made as endemic typhus using the Weil Felix test.[12]

Reports from post 2000s

More epidemiological data became available from Sri Lanka in the 21st century, after local researchers started collaborative work with different international groups, including Centers for Disease Control and Prevention (CDC), Atlanta (USA) and Unite´ des Rickettsies, Marseille, France. Scrub typhus, spotted fever group rickettsioses, endemic typhus and Q fever have been demonstrated in Sri Lanka.[15,16,17]

Geographical distribution of rickettsioses in Sri Lanka

Geographical distribution of rickettsioses in Sri Lanka is varied and is possibly associated with the distribution of vectors and reservoir animals.

The Central Province has reported a predominance of spotted fever rickettsioses. Distribution of spotted fever rickettsioses in the Central Province was mainly found in the western slopes of the central hills such as Kegalle, Mawanella and the northern parts of the province, up to

Nawalapitiya. Mawanella, Kegalle, Gampola and Nawalapitiya hospitals as well as the major tertiary care centre for these areas, Teaching Hospital, Peradeniya has a regular influx of patients with rickettsioses.[15,17,18,19,20] Serological evidence of scrub typhus and murine typhus has also been reported to a lower extent.[15,19]

The Western Province has predominantly reported patients with scrub typhus, while serological evidence for spotted fever has also been present.[16]

The Southern Province has a mix of rickettsioses. A hospital based study conducted in District General Hospital, Matara in 2009 identified 25 (14%) patients with scrub typhus, 6 (3%) with SFG rickettsioses, 3 (1.6%) with acute Q fever, 3 (1.6%) with murine typhus and 3 (1.6%) infected by *Rickettsia felis*.[21] A study conducted at Teaching Hospital, Karapitiya where 883 paired sera were analyzed, identified 17.7% with confirmed rickettsioses. Spotted fever was the most common type of rickettsioses identified in this study.[22]

The North Western Province predominantly has scrub typhus, as reported by serological evidence from patients admitted to Provincial General Hospital, Kurunegala and Base Hospital, Dambadeniya.[18,23]

The North Central Province has been traditionally linked with scrub typhus. In an island wide surveillance study that used IFA and ELISA as testing methods, patients admitted to Provincial General Hospital, Anuradhapura were identified to have predominantly scrub typhus.[18]

The Northern Province has a predominance of scrub typhus.[24] However, an outbreak attributed to spotted fever, diagnosed by sero-positivity to *Rickettisa conorii* using IFA has been reported from army personnel within the Kilinochchi area from October to December 2012.[25] Premaratne *et al* reported that while scrub typhus was the predominant rickettsioses found among army personnel deployed in the Northern Province presenting with an acute febrile illness, the majority of apparently healthy army personnel had serological evidence of exposure to spotted fever.[26]

Sero-prevalence of rickettsioses among the community has not been studied that well in Sri Lanka. In a study conducted involving healthy volunteers from Kandy, a suburb of Mawanella (Hemmathagama), Mahiyanganaya and Tissa-Kataragama, sero-positivity as defined by an IFA titre of $\geq 1/64$ has been reported for both *R. conorii* and *O. tsutsugamushi* in all areas.[27]

The studies mentioned above used different testing methods, including IFA and ELISA. Most studies were from single hospitals or included a number of hospitals with voluntary participation. Further, the cut off points used in different studies were different and a single

study including all provinces and districts in a systematic manner has not been conducted in Sri Lanka.

Rickettsioses in travellers returning from Sri Lanka

Sri Lanka is a prime travel destination currently. The demography of travelers arriving in Sri Lanka varies and many of them are engaged in outdoor activities that increase their risk of being bitten by ticks and mites. Rickettsial infections in returning travelers have been reported from France, Norway and Australia.[28,29,30] This is also an issue for Sri Lankans domiciled abroad who return after visiting relatives.[29] In some instances, there have been delays in diagnosing this infective disease. However, as more epidemiological studies and case reports emerge from Sri Lanka, the global community will become aware of Sri Lanka as a hot-spot for rickettsioses and these delays in diagnosis could be minimized.

Potential pathogens causing rickettsioses in Sri Lanka

Identification of the aetiological agent with serology remains problematic due to the high cross reactivity of antibodies. Isolation of the organism in cell cultures or molecular identification is needed for this purpose. Only a few studies have attempted to identify the aetiological agent causing rickettsioses in Sri Lanka using these methods.

Orientia tsutsugamushi strains found from patients in Sri Lanka have identified the presence of all major genotypes. This study was conducted using PCR followed by sequencing of the 56kD protein gene present in eschar and buffy coat samples of patients. Karp [Thai-related] genotypes were the most frequent followed by Kato-related, Kawasaki-related and Gilliam-related genotypes. The authors also noted a close homology of one strain with the Kuroki-Boryong organism. This work highlights the diversity of the *Orientia tsutsugamushi* strains in Sri Lanka.[31]

Rickettsia sibirica mongolitimonae has been demonstrated in a tissue sample obtained from an eschar using sequencing of the *ompA* gene from a traveler returning to France following a visit to Sri Lanka.[28]

There has also been a report where the species identified from Sri Lanka was found to be most similar to *Candidatus Rickettsia kellyi*, previously reported from Tamil Nadu, India.[32]

However, this remains a research priority for the country as identification of the exact aetiology or aetiologies causing rickettsioses in Sri Lanka will enable implementation of specific control measures as well as to develop more specific diagnostic tests.

Clinical presentation of rickettsioses

Rickettsial infections are traditionally considered to be fevers with rashes with an inoculation eschar. However, presentation of rickettsioses can be non-specific with a wide range of clinical symptoms, which makes it difficult to distinguish this infection clinically from other common viral illnesses.[22] This has been reported in Sri Lanka during an outbreak of chikungunya infections.[33]

In studies conducted in Sri Lanka, inoculation eschar has been mostly associated with scrub typhus.[21] The proportion of patients with confirmed scrub typhus with eschar ranged from 25% to 89%.[16,21,23] Rash is generally associated with a lesser proportion of patients with scrub typhus, sometimes being absent.[21,23]

Spotted fever rickettsioses in Sri Lanka presents predominantly with fever and rash. The proportion of patients with spotted fever presenting with rash has varied from 16% to 100%.[21,34] Eschar is rarely associated with spotted fever.[18,21,24] However, in the outbreak of rickettsioses in the Kilinochchi area mentioned above, a considerable proportion of patients with serological evidence of spotted fever had demonstrated an eschar like lesion, particularly in the eye.[25]

The rash in spotted fever is typically a macular papular rash. In a study conducted at Teaching Hospital, Peradeniya, including 134 patients with serum IgG >1/256 and IgM 1/32, the commonest type of rash identified was a discrete macular papular rash of erythematous hue involving the arms and forearms. Involvement of palms and soles had been noted in about 50% of the study group.[34] Variations in the skin involvement has been described in Sri Lanka, and it is important for treating clinicians to be aware of these differences in order to initiate prompt treatment. Alternative types of rashes that have been reported include fern-leaf pattern skin necrosis[35] and purpura fulminans.[36]

Neurological manifestations may also be associated with rickettsioses. This has been reported in Sri Lanka in association with spotted fever and scrub typhus. Neurological signs in patients with spotted fever have been particularly present among elderly patients. The reported neurological features range from altered consciousness, tremors, rigidity and dyskinesia. According to Kularatne et al, most of the features resolved after treatment with appropriate antibiotics. The authors also state that EEG changes compatible with encephalitis was noted among some patients.[37] Extra pyramidal involvement maybe marked enough to make the presentation very similar to Parkinsonism.[38] Another neurological manifestation that has been attributed to rickettsial infections is facial nerve palsy following intra-aural tick bites.[39]

Joint involvement leads to many patients presenting with joint pains of large and small joints. This has been reported from patients with spotted fever rickettsioses.[20] Patients who present with predominant features of joint involvement, particularly with major joint arthritis have also been reported.[40] Patients with small joint involvement and other features such as oral ulceration and hair loss may lead to an initial impression of connective tissue disorders.[41]

Atypical presentations and complications have been reported among patients with scrub typhus too. Scrub typhus implicated in acute hearing loss has been reported from the Western Province.[42] Patients with scrub typhus presenting with fever and late onset diarrhoea mimicking enteric fever have also been reported.[43] Pancytopaenia secondary to haemophagocytosis has also been reported.[44]

Association with climate and weather conditions

An overview of rickettsioses in Sri Lanka shows that the dry regions of the country with scrub jungles has a predominance of scrub typhus while the hilly areas have a predominance of spotted fever.[18] However, exceptions to this has also been reported.

In the North Western Province, the number of cases identified has been higher during the dry season.[23] This may be associated with the seasonal variations of vector density, or behavioral changes associated with the dry season that increases vector human contact. However, a study conducted at the Teaching Hospital, Karapitiya has identified that more patients present during the rainy season.[22] These differences may be due to the behavioral traits of the associated vectors.

Vectors and host animals

While the exact host animals or vectors have not been pinpointed in Sri Lanka, serological evidence for rickettsioses has been found among domestic and wild animals in the country. Most patients with serological evidence of rickettsial infections have association with domestic or wild animals and report contact with potential vectors such as ticks.[20]

Goat and cattle sera obtained from Sri Lanka have been shown to contain antibodies against spotted fever group rickettsial agents, including *Rickettsia conorii*, in addition to antibodies against *Coxiella burnetti*.[45] More recently, serological evidence for exposure to *R. conorii* and *Orientia tsutsugamushi* has been identified among dogs from Kandy, Thambavita, western slope of central hills and Unawatuna, with areas from the western slopes of central hills demonstrating the highest rate of sero-prevalence.[46] Small mammals, namely *Rattus rattus*, *Bandicota indica* and *Mus fernandoni*, captured from Kandy and Kurunegala districts have been identified to have rickettsiae in their blood using qPCR.[47]

Ticks of the *Rhipicephalus* species removed from a patient with demonstrable sero conversion to rickettsial antibodies has been shown to have rickettsial DNA.[39] A number of ticks associated with rickettsial infections have been recovered from both humans (autocariasis) and animals in Sri Lanka. Interestingly, these ticks are known to reside on domestic as well as wild animals.[48] PCR based testing for 17 kDa antigen of spotted fever rickettsiae has identified these to be present in ticks found on animals in Sri Lanka. These have been found in three *Amblyomma* species: *Amblyomma testudinairum* found on a wild boar, *Amblyomma clypeolatum* found on a star tortoise, and *Amblyomma javanense* found on a pangolin and *Rhipicephalus sanguineus* found on a dog.[48] All species except *A. javanense* have been known to infect humans and therefore can contribute to the spread of rickettsioses in Sri Lanka.

Ectoparasites present on *Suncus murinus, Bandicota indica* and *Golunda ellioti,* captured from Kurunegala and Kandy districts have demonstrated to have rickettsiae. These ectoparasites included *Rhipicephalus haemaphysaloides*, *Ixodes ceylonensis, Haemaphysalis spinigera*, *Haemaphysalis sp.*, *Stivalius aporus* and *Xenopsylla cheopes.*[47] Japanese researchers have identified rickettsiae in *Amblyomma trimaculatum* ticks found as ecto-parasites on snakes (*Boiga forsteni*) from Sri Lanka.[49]

Potential for further research and development

Rickettsioses are a group of notifiable diseases in Sri Lanka. However, most patients are diagnosed on clinical suspicion, and in the absence of laboratory confirmation, identification of the actual disease burden is problematic.

Sri Lanka does not have sufficient laboratory facilities to cater to all hospitals that treat patients with rickettsioses. Clinicians in regions where rickettsioses are endemic commence treatment with appropriate antibiotics on clinical suspicion. In a study conducted on 28 febrile patients, after 7 days of hospitalization without a confirmed diagnosis and who responded rapidly to doxycycline, 21 had shown serological evidence of rickettsioses.[50]

While use of paired sera to demonstrate a rise in antibody titre is the gold standard for diagnosis of rickettsioses, it is practically difficult in clinical settings. Establishment of a cut off value to interpret a single antibody titre detected by IFA is also a research priority. Using a cut off level of >1/256 for IgG for diagnosis of spotted fever has been associated with a false positive rate of 11.3% and a false negative rate of 4.3% among those with illness for > 7 days. The same cut off has been associated with a 0% false positive rate and a 12% false negative rate among those with illness for < 7 days. For scrub typhus, the same cut off has had a false positive rate of 2% and a false negative rate of 0% among those with illness for > 7 days, and a false positive rate of 0% and a false negative rate of 14.2% among those with illness for < 7 days.[51] However, these values need to be regionally validated. This is difficult in the context of

high seroprevalence among healthy individuals.[27] Locally validated algorithms including clinical features, IFA titres as well as duration of illness may be the way forward.

The increasing interaction of wild animals such as monkeys, peacocks and wild boar with domestic animals and humans may lead to an increase in rickettsial infections.[48] Mapping out the ecological niche of vectors and hosts of rickettsial infections is also a need.

While there have been remarkable improvements in rickettsiology in Sri Lanka during the past decade, much remains to be elucidated. A comprehensive approach involving public health officials, veterinarians, medical doctors, zoologists and environmentalists with a one health approach is essential for the further development of the field.

References

1. Walker DH. Rickettsiae and rickettsial infections: the current state of knowledge. *Clin Infect Dis*. 2007; 45 (Suppl 1):S39-44. *doi: https://doi.org/10.1086/518145*
2. La Scola B, Raoult D. Laboratory diagnosis of rickettsioses: current approaches to diagnosis of old and new rickettsial diseases. *J Clin Microbiol*. 1997; 35(11):2715-27. Review. PubMed PMID: 9350721; PMCID:PMC 230049.
3. Faccini-Martínez ÁA, García-Álvarez L, Hidalgo M, Oteo JA. Syndromic classification of rickettsioses: an approach for clinical practice. *Int J Infect Dis*. 2014; 28:126-39. *doi: 10.1016/j.ijid.2014.05.025*.
4. Portillo A, Santibáñez S, García-Álvarez L, et al. Rickettsioses in Europe. *Microbes Infect*. 2015; 17(11-12):834-8. *doi: 10.1016/j.micinf.2015.09.009*.
5. Raoult D, Roux V. Rickettsioses as paradigms of new or emerging infectious diseases. *Clin Microbiol Rev*. 1997; 10(4):694-719. *doi: https://doi.org/10.1128/CMR.10.4.694*
6. Parola P, Paddock CD, Raoult D. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. *Clin Microbiol Rev*. 2005; 18(4):719-56 *doi: https://doi.org/10.1128/CMR.18.4.719-756.2005*
7. Kelly DJ, Fuerst PA, Ching WM, Richards AL. Scrub typhus: the geographic distribution of phenotypic and genotypic variants of *Orientia tsutsugamushi*. *Clin Infect Dis*. 2009; 15;48 (Suppl 3):S203-30. *doi: https://doi.org/10.1086/596576*
8. Izzard L, Fuller A, Blacksell SD, et al. Isolation of a novel Orientia species (*O. chuto* sp. nov.) from a patient infected in Dubai. *J Clin Microbiol*. 2010; 48(12):4404-9. *doi: 10.1128/JCM.01526-10.*
9. Masakhwe C, Linsuwanon P, Kimita G, et al. Identification and characterization of *Orientia chuto* in trombiculid chigger mites collected from wild rodents in Kenya. *J Clin Microbiol*. 2018; 56(12):e0 1124-18. *doi:10.1128/JCM.01124-18.*
10. Kelly DJ, Richards AL, Temenak J, et al. The past and present threat of rickettsial diseases to military medicine and international public health. *Clin Infect Dis*. 2002 15;34(Suppl 4):S145-69. *doi: https://doi.org/10.1086/339908*
11. Premaratne. Ranjan. Rickettsial infections in Sri Lanka: yesterday, today, tomorrow. P.B. Fernando Oration 2010. *Journal of the Ceylon College of Physicians*. 2011; 42:11-15

No doi
12. Vasanthathilaka VWJK, Senanayake N. Typhus fever from the mountainous areas in Central Sri Lanka. *Kandy Medical Jounal*.1999; 5:4:6-9. *No doi*
13. Van Peenen PF, See R, Soysa PE, Irving GS. Sero-epidemiological survey of hospital-associated populations in Colombo, Sri Lanka. *Southeast Asian J Trop Med Public Health*. 1976; 1:16-20. *No doi*
14. Kovácová E, Sixl W, Stünzner D, et al. Serological examination of human and animal sera from six countries of three continents for the presence of rickettsial antibodies. *Eur J Epidemiol*. 1996; 12(1):85-9. *doi: http://dx.doi.org/10.1007/BF00144434*
15. Kularatne SAM, Edirisingha JS, Gawarammana IB, et al. Emerging rickettsial infections in Sri Lanka: the pattern in the hilly Central Province. *Trop Med Int Health*. 2003; 8(9):803-11. *doi: https://doi.org/10.1046/j.1365-3156.2003.01108.x*
16. Premaratna R, Loftis AD, Chandrasena TG, et al. Rickettsial infections and their clinical presentations in the Western Province of Sri Lanka: a hospital-based study. *Int J Infect Dis*. 2008; 12(2):198-202. *doi: https://doi.org/10.1016/j.ijid.2007.06.009*
17. Nagalingam K, Rolain JM, Thevanesam V, et al. Spotted fever rickettsioses in children in Sri Lanka. *Clin Microbiol Infect*. 2009; 15 (Suppl 2):330-1. *doi: 10.1111/j.1469-0691.2008.02262.x.*
18. Liyanapathirana VC, Thevanesam V. Seroepidemiology of rickettsioses in Sri Lanka: a patient based study. *BMC Infect Dis*. 2011; 11:328. *doi:10.1186/1471-2334-11-328.*
19. Kularatne SAM, Rajapakse RP, Wickramasinghe WM, et al. Rickettsioses in the central hills of Sri Lanka: serological evidence of increasing burden of spotted fever group. *Int J Infect Dis*. 2013; 17(11):e988-92. *doi: 10.1016/j.ijid.2013.05.014.*
20. Weerakoon KG, Kularatne SAM, Rajapakse J, et al. Revisiting clinico-epidemiological pattern of human rickettsial infections in the central region of Sri Lanka: a hospital based descriptive study. *BMC Res Notes*. 2017; 10(1):400. *doi: 10.1186/s13104-017-2727-1*.
21. Angelakis E, Munasinghe A, Yaddehige I,. Detection of rickettsioses and Q fever in Sri Lanka. *Am J Trop Med Hyg*. 2012; 86(4):711-2. *doi: 10.4269/ajtmh.2012.11-0424*.
22. Reller ME, Bodinayake C, Nagahawatte A, et al. Unsuspected rickettsioses among patients with acute febrile illness, Sri Lanka, 2007. *Emerg Infect Dis*. 2012; 18(5):825-9. *doi: 10.3201/eid1805.111563*.
23. De Silva N, Wijesundara S, Liyanapathirana V, et al. Scrub typhus among pediatric patients in Dambadeniya: a base hospital in Sri Lanka. *Am J Trop Med Hyg*. 2012; 87(2):342-4. *doi: 10.4269/ajtmh.2012.12-0170*.
24. Pradeepan JA, Ketheesan N, Murugananthan K. Emerging scrub typhus infection in the northern region of Sri Lanka. *BMC Res Notes*. 2014; 7:719. *doi:10.1186/1756-0500-7-719.*
25. Dahanayaka N.J., Semage S.N., Weerakoon K.G.,. An unusual outbreak of Rickettsial infection among army soldiers engaged in reconnaissance mission in Northern Sri Lanka. *Ceylon Medical Journal* 2017; 62(2), pp.108–109. *doi: http://doi.org/10.4038/cmj.v62i2.8477*
26. Premaratna R, Ariyaratna N, Attanayake C, et al. Rickettsial infection among military personnel deployed in Northern Sri Lanka. *BMC Infect Dis*. 2014; 14:3864. *doi: 10.1186/s12879-014-0688-8*

27. Nanayakkara DM, Kularatne SAM, Wickramasingh WMRS,. Seroprevelence of rickettsioses among human population of Sri Lanka: A study in four regions. *Proceedings of the Peradeniya University Research Sessions, Sri Lanka*. 2009; 14:65–67.
Available at http://dlib.pdn.ac.lk/handle/1/4438. Accessed on 28/12/2018
28. Cordier, C., P. Tattevin, C. Leyer, M. et al. "Rickettsia Sibirica Mongolitimonae Infection, Sri Lanka". *The Journal of Infection in Developing Countries.* 2017; 11(08):668-71.
doi: https://doi.org/10.3855/jidc.8743
29. Jensenius M, Montelius R, Berild D, Vene S. Scrub typhus imported to Scandinavia. *Scand J Infect Dis.* 2006; 38(3):200-2. *doi: https://doi.org/10.1080/00365540500277342*
30. Stokes PH, Walters BJ. Spotted fever rickettsiosis infection in a traveler from Sri Lanka. *J Travel Med*. 2009; 16(6):436-8. *doi:10.1111/j.1708-8305.2009.00360.x.*
31. Premaratna R, Blanton LS, Samaraweera DN, et al. Genotypic characterization of *Orientia tsutsugamushi* from patients in two geographical locations in Sri Lanka. *BMC Infect Dis*. 2017; 17(1):67. *doi: 10.1186/s12879-016-2165-z.*
32. Nanayakkara DM, Serological detection and identification of Rickettsial species infecting humans. MPhil Thesis. Postgraduate Institute of Science, University of Peradeniya, Sri Lanka. 2011. Available from
http://dlib.pdn.ac.lk/bitstream/123456789/2973/1/Nanayakkara%202011.pdf. Last accessed on 31/12/2018.
33. Premaratna R, Halambarachchige LP, Nanayakkara DM, et al. Evidence of acute rickettsioses among patients presumed to have chikungunya fever during the chikungunya outbreak in Sri Lanka. *Int J Infect Dis*. 2011; 15(12):e871-3. *doi: 10.1016/j.ijid.2011.09.010.*
34. Weerakoon K, Kularatne SA, Rajapakse J, et al. Cutaneous manifestations of spotted fever rickettsial infections in the Central Province of Sri Lanka: a descriptive study. *PLoS Negl Trop Dis*. 2014; 8(9):e3179. *doi: 10.1371/journal.pntd.0003179*.
35. Luke N, Munasinghe H, Balasooriya L, Premaratna R. Widespread subcutaneous necrosis in spotted fever group rickettsioses from the coastal belt of Sri Lanka- a case report. *BMC Infect Dis.* 2017; 17(1):278. *doi: 10.1186/s12879-017-2375-z.*
36. Dalugama C, Gawarammana IB. Rare presentation of rickettsial infection as purpura fulminans: a case report. *J Med Case Rep*. 2018; 12(1):145. *doi: 10.1186/s13256-018-1672-5.*
37. Kularatne SAM, Weerakoon KG, Rajapakse RP, et al A case series of spotted fever rickettsiosis with neurological manifestations in Sri Lanka. *Int J Infect Dis.* 2012 ;16(7):e514-7. *doi: 10.1016/j.ijid.2012.02.016.*
38. Premaratna R, Wijayalath SH, Miththinda JK,. Scrub typhus mimicking Parkinson's disease. *BMC Res Notes*. 2015; 8:438. *doi: 10.1186/s13104-015-1428-x.*
39. Kularatne SAM, Fernando R, Selvaratnam S, et al. Intra-aural tick bite causing unilateral facial nerve palsy in 29 cases over 16 years in Kandy, Sri Lanka: is rickettsial aetiology possible? *BMC Infect Dis*. 2018; 18(1):418. *doi: 10.1186/s12879-018-3338-8.*
40. Premaratna R, Chandrasena TG, Rajapakse RP,. Rickettsioses presenting as major joint arthritis and erythema nodosum: description of four patients. *Clin Rheumatol*. 2009; 28(7):867-8. *doi: 10.1007/s10067-009-1166-3*.
41. Premaratna R, Liyanaarachchi E, Rajapakse R, et al. A patient with spotted fever group rickettsiosis mimicking connective tissue disease. *Ceylon Medical Journal*. 2012;57(3):127–128. *doi: http://doi.org/10.4038/cmj.v57i3.4707*

42. Premaratna R, Chandrasena TG, Dassayake AS, et al. Acute hearing loss due to scrub typhus: a forgotten complication of a reemerging disease. *Clin Infect Dis*. 2006; 42(1):e6-8. *doi: https://doi.org/10.1086/498747*
43. Premaratna R, Nawasiwatte BM, Niriella MA, et al. Scrub typhus mimicking enteric fever; a report of three patients. *Trans R Soc Trop Med Hyg*. 2010; 104(4):309-10. *doi: 10.1016/j.trstmh.2009.08.011*
44. Premaratna R, Williams HS, Chandrasena TG, et al. Unusual pancytopenia secondary to haemophagocytosis syndrome in rickettsioses. *Trans R Soc Trop Med Hyg.* 2009; 103(9):961-3. *doi: 10.1016/j.trstmh.2009.04.003*.
45. Kovácová E, Sixl W, Stünzner D, Urvölgyi J, Kazár J. Serological examination of human and animal sera from six countries of three continents for the presence of rickettsial antibodies. *Eur J Epidemiol*. 1996;12(1):85-9. *No doi*
46. Nanayakkara DM, Rajapakse RP, Wickramasinghe S, Kularatne SA. Serological evidence for exposure of dogs to *Rickettsia conorii, Rickettsia typhi*, and *Orientia tsutsugamushi* in Sri Lanka. *Vector Borne Zoonotic Dis*. 2013; 13(8):545-9. *doi: 10.1089/vbz.2012.1049.*
47. Yathramullage S, Rajapakse J, Boyagoda S. Rickettsiae reservoirs among small mammals (rats, mice and shrews) and their arthropod vectors in Sri Lanka. *Ceylon Journal of Science.* 2018: 47(2):175–183. *doi: http://doi.org/10.4038/cjs.v47i2.7514*
48. Liyanaarachchi DR, Rajakaruna RS, Dikkumbura AW, Rajapakse RP. Ticks infesting wild and domestic animals and humans of Sri Lanka with new host records. *Acta Trop*. 2015; 142:64-70. *doi: 10.1016/j.actatropica.2014.11.001*.
49. Andoh M, Sakata A, Takano A, et al. Detection of Rickettsia and Ehrlichia spp. in ticks associated with exotic reptiles and amphibians imported into Japan. *PLoS One*. 2015; 10(7):e0133700. *doi: 10.1371/journal.pone.0133700.*
50. Premaratna R, Rajapakse RP, Chandrasena TG, et al. Contribution of rickettsioses in Sri Lankan patients with fever who responded to empirical doxycycline treatment. *Trans R Soc Trop Med Hyg*. 2010; 104(5):368-70. *doi: 10.1016/j.trstmh.2009.10.006.*
51. Premaratna R, Weerasinghe S, Ranaweera A, et al. Clinically helpful rickettsial disease diagnostic IgG titers in relation to duration of illness in an endemic setting in Sri Lanka. *BMC Res Notes*. 2012; 5:662. *doi: 10.1186/1756-0500-5-662.*

Research article

Bacteria mediated silver nanoparticles: comparison as potent antibiofilm agents

M Peiris[1], N Fernando[1], P Jayaweera[2], M Weerasekera[1], C Gunasekara[1,3]

Sri Lankan Journal of Infectious Diseases 2019 Vol.9 (1):13-23

DOI: http://dx.doi.org/10.4038/sljid.v9i1.8227

Abstract

Introduction: Biosynthesized silver nanoparticles (AgNPs) have good antimicrobial properties comparable to chemically synthesized silver nanoparticles. Further, they have good potential as antibiofilm agents.

Methods: AgNPs were synthesized from *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus (*ATCC 25923) and *Acinetobacter baumannii* (confirmed clinical isolate) and physically characterized by several techniques. The antibiofilm activity of the AgNPs against biofilms of *P. aeruginosa*, *S. aureus* and *C. albicans* was studied using crystal violet assay. Biofilms were formed in 96-well polystyrene plates and treated with biosynthesized AgNPs for 24 and 48 h.

Results: AgNPs synthesized by all bacteria except *S. aureus* mediated AgNPs displayed 50% biofilm inhibition at AgNP concentrations between 1.98 - 0.225 mg/ml. *S. aureus* mediated AgNPs showed 50% biofilm inhibition only against *S. aureus* biofilm. Scanning Electron microscopic images indicated that biosynthesized AgNPs were able to decrease surface coverage of biofilms and to reduce the extracellular matrix causing morphological changes in biofilms noticeably.

Conclusion: This study reports the antibiofilm activity of bacteria mediated AgNPs. This is the first report on antibiofilm activity of AgNPs synthesized by *Acinetobacter baumannii* and also as a comparison of antibiofilm activity of several bacteria mediated AgNPs. According to the

---

[1]*Department of Microbiology, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka*
[2]*Department of Chemistry, Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka*
[3]*Centre for Plant Materials and Herbal Products Research, University of Sri Jayewardenepura, Sri Lanka*

Address for correspondence: Dr Chinthika P Gunasekara, Department of Microbiology, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka Telephone: +94718262860

Email: chinthika@sjp.ac.lk *https://orcid.org/0000-0002-6003-3088*

Received 18 August 2018 and accepted 6 October 2018

results, low dosages of green AgNPs can be applied in treating drug-resistant microbial infections in a cost effective manner. In conclusion, the bacterial synthesized AgNPs have antibiofilm activity and good stability suggesting its usefulness as economic and environmental friendly antibiofilm agents.

Keywords: Biofilms, Biosynthesis, Silver nanoparticles, Extracellular polysaccharides, Pseudo hyphae

Introduction

Bacterial biofilms pose a major challenge in medicine and are an important contributory factor towards the resistance of bacteria to antimicrobial treatment. Biofilms are a consortium of bacteria, fungi or other microorganisms which are embedded in a polysaccharide matrix where they can multiply, communicate with each other and persist, thus leading to biofilm infections specially associated with medical devices such as indwelling catheters[1, 2], contact lenses[3] and prosthetic heart valves.[4] Biofilms are responsible for disease outbreaks. Medically important biofilm forming pathogens include *Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter baumannii and Candida albicans.*

It is a well-established fact that biofilm organisms show greater resistance to antibiotics. Further, the biofilm formation is affected by the level of mutations and quorum-sensing mechanisms which results in antibiotic resistance leading to more virulent strains.[5] Biofilms are more difficult to be penetrated through the polysaccharide matrix by even high concentrations of antibiotics. These biofilms have defensive mechanisms against antibiotics and antifungals under adverse conditions. Therefore development of novel antibiofilm agents is important in combating biofilm related infections.

Nanoparticles are emerging as novel antimicrobial agents and are widely used in various applications. Metal nanoparticles such as silver (AgNPs)[6,7], titanium dioxide[7] have been reported to be effective against some biofilm forming bacteria indicating their potential for future development as effective biofilmicidal agents. AgNPs show a broad spectrum of bactericidal effects.[8] Martinez-Gutierrez[9] reported the inhibition of biofilm formation by AgNPs as a strategy to contain biofilm formation in medical devices. Due to the cost of production and toxicity of chemically synthesized AgNPs, biosynthesis of AgNPs involving bacteria is being considered as an attractive alternative. Bacterial enzymes act as reducing agents of Ag^+ ions.[10] Highly stable AgNPs can be produced from bacteria such as *P. aeruginosa*[11] and *Acinetobacter calcoaceticus*.[12]

In this study we have investigated the potential antibiofilm ability of bacterial biosynthesized silver nanoparticles *in vitro*.

Materials and methods

Microbial cultures

The bacterial strains *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), *S. aureus (*ATCC 25923), *A. baumannii* (confirmed clinical isolate) and *Candida albicans (*ATCC 10231) were obtained from the culture collection (Bacteriology laboratory, Department of Microbiology) and cultivated in Nutrient Broth Medium.

Ag nanoparticle biosynthesis

P. aeruginosa (ATCC 27853), *E. coli* (ATCC 25922), *A. baumannii* (confirmed clinical isolate) and *S. aureus* (ATCC 25923) were cultured in Nutrient Broth Medium (Himedia, India) and incubated for 72 h at 37 °C while shaking at 200 rpm. After the incubation period, the cultures were centrifuged at 6000 rpm for 20 min and the supernatants were collected.[10] Aqueous silver nitrate solution (1 M, Park, UK) was added into culture supernatants to prepare a final concentration of 0.4 g/l and the mixture was kept at 60 °C to induce AgNP formation.

Biosynthesis of AgNPs was confirmed by UV–Visible Spectrophotometry. The AgNPs were diluted ten times and UV Visible absorption was measured using a UV-Visible spectrophotometer (PerkinElmer Lambda 35, USA). Transmission Electron Microscopy (TEM) was used to determine the particle size distribution of AgNPs (JEOL JEM 2100, Japan).

Preparation of microbial biofilms

Preparation of standard cell suspension (1.0 McFarland standard)

Colonies of 24 h old cultures of *C. albicans*, *S. aureus* and *P. aeruginosa* were inoculated in Brain Heart Infusion broth (Himedia, India) separately and incubated at 37 °C for 24 h. After 24 h of incubation, cell pellets were harvested by centrifugation at 3000 rpm for 10 min. Cell pellets were washed twice with PBS. An optical density (OD) of 0.1 (corresponding to 1×10^8 cells/ml) was obtained at 595 nm (Multiskan EX, Thermo Scientific, USA) as the standard cell suspensions for each organism.

Formation of 48 h old biofilms

Two hundred microliters of standard cell suspensions were added into each well and incubated for 24 h at 37 °C. After 24 h of incubation, 100 µl of the medium was removed and the well was replenished with 100 µl of fresh BHI medium followed by a further incubation at 24 h at 37 °C.

Assessment of biofilm formation

To interpret the biofilm production ability of the selected pathogens, a standard criteria described by Stepanovic et al.[13] was applied as follows. The optical density of the cut off was calculated based on the OD of the negative control.

Optical density cut-off value (ODc) = average OD of negative control (BHI only) + 3× standard deviation (SD) of negative control.

Average OD value	**Biofilm production**
≤ ODc / ODc < ~ ≤ 2x ODc	Non/weak
2x ODc < ~ ≤ 4x ODc	Moderate
> 4x ODc	Strong

Determination of Inhibitory concentrations for 48 hour mature biofilms

The 48 h old mature biofilms were washed twice with sterile PBS. Then 100 µl of serially diluted nanoparticle solutions were added into the corresponding wells in the microtiter plate. Negative control wells consisted of non-treated biofilms or sterile BHI. $AgNO_3$ (0.5% w/v) treated biofilms were used as positive controls. The plates were incubated at 37 °C for 24 h. The wells were washed twice with 200 µl of sterile PBS gently to remove free floating planktonic bacteria and biofilm viability was determined using Crystal violet assay. The biofilms were fixed with 200 µl of sodium acetate (2% w/v) and stained with 200 µl of crystal violet dye (0.1% w/v). Excess stain was rinsed off by thorough washing with sterile distilled water and plates were allowed to dry. After drying, 200 µl of 30% acetic acid was added into each well. The wells were incubated for 30 min. Acetic acid was aspirated and transferred to a new plate. The absorbance was measured at 595 nm using an ELISA plate reader and the percentage of biofilm inhibition calculated using the following equation. The experiment was carried out in triplicate, the data averaged, and standard deviation calculated.

$$\text{Percentage of biofilm inhibition} = \frac{(OD_{595} \text{ of untreated biofilm} - OD_{595} \text{ of treated biofilm})}{OD_{595} \text{ of untreated biofilm}} \times 100$$

Scanning Electron Microscopy (SEM)

The biofilms were cultured on 1 cm diameter sterile coverslips pretreated with conc. H_2SO_4 and absolute ethanol in 24 well culture plates (Corning, Germany) for 48 h and medium was replenished at 24 h of incubation. AgNPs were treated after 48 hours and further incubated for 24 h at 37 °C. Formed biofilms were fixed with 2.5% glutaraldehyde and washed with sterile PBS. Dehydration of samples was followed using an alcohol series (30%, 50%, 60%, 70%, 80%, 90%, 95% and absolute ethanol. After overnight drying in a desiccator, samples were coated with gold before imaging using a ZEISS EVO 18 Scanning Electron Microscope (Germany).

Results

The biosynthesis of AgNPs from all four bacteria were confirmed by the presence of narrow absorbance peaks observed approximately near 420 nm wavelength (Fig. 1). *S. aureus* mediated AgNPs gave a broad peak ranging from 420-450 nm.

According to TEM particle size analysis, all AgNPs were spherical in shape. The average sizes of the NPs were 11.14 ± 6.59 nm (S-AgNPs), 11.71 ± 2.73 nm (P-AgNPs), 12.87 ± 2.95 nm (E-AgNPs) and 12.22 ± 2.45 nm (A-AgNPs). Comparatively smaller AgNPs were produced by *S. aureus* and *P. aeruginosa*. Transmission electron microscopy identified all four AgNP species with average particle size < 20 nm in diameter suggesting the possibility of strong antimicrobial activity due to the small particle size.

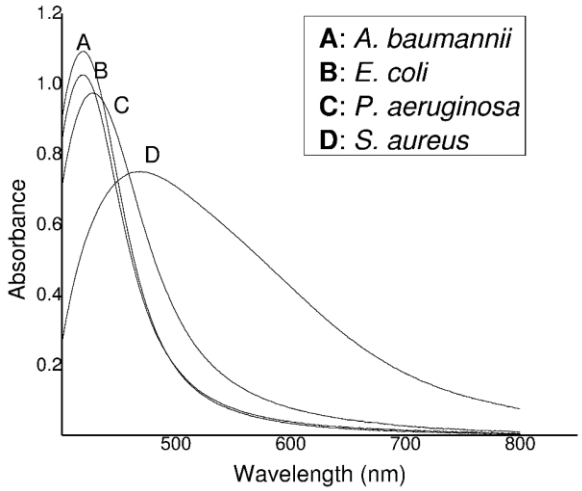


Fig. 1 UV-Visible absorption spectra of biosynthesized AgNPs

At the optimized conditions, the AgNP concentrations synthesized by *A. baumannii* (A-AgNPs)(0.665 mg/ml), *E. coli* (E-AgNPs)(0.99 mg/ml), *P. aeruginosa* (P-AgNPs)(0.45 mg/ml) and *S. aureus* (S-AgNPs) (0.435 mg/ml) were calculated by obtaining the dry weight.

Biofilm formation

Biofilm formation of tested bacterial strains was determined using the crystal violet assay. Fig. 2 represents the biofilm formation assay at 24 h and 48 h. All three organisms were found to form strong biofilms according to the criteria specified by Stepanović et al.[13] *P. aeruginosa* and *S. aureus* produced the strongest biofilms followed by *C. albicans* at both time points.

Bacterial biofilm formation was further confirmed using SEM imaging. Scanning electron microscopy

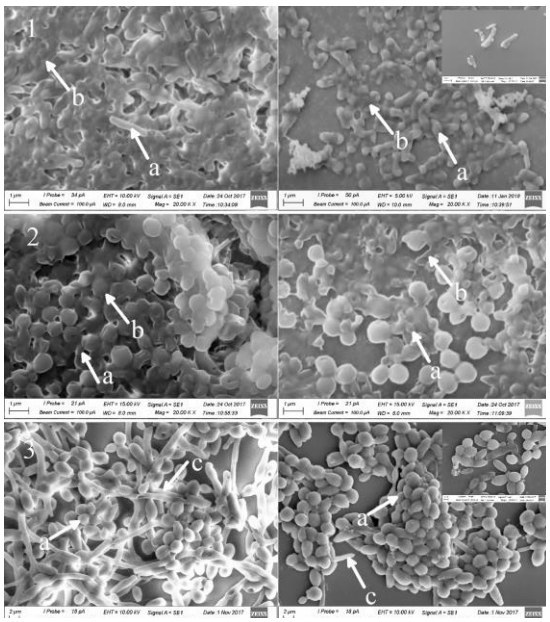


Fig. 2 SEM images of Biofilms of 1) *P. aeruginosa*, 2) *S. aureus* and 3) *C. albicans* **Left**: Before and **Right**: after treatment with AgNPs produced *by S. aureus* (a) cells, (b) extracellular matrix, (c) pseudo hyphae

of *P. aeruginosa* and *S. aureus* biofilms demonstrated a network of thick extracellular matrix surrounding abundant bacterial cells which suggests biofilm formation. The biofilm architecture included presence of interconnecting thread like structures, possibly formed due to water channels between the biofilm embedded cells. *C. albicans* biofilms demonstrated budding oval yeast cells with extensive production of pseudo hyphae and presence of budding scars. (Fig. 2)

Biofilm inhibition by AgNPs

Serial dilutions of bacterial synthesized AgNPs were prepared and used to treat 24 h old bacterial biofilms. Biofilm treatment was carried out for 24 h and 48 h. Highest inhibition of bacterial biofilms were observed at the highest tested AgNP concentrations of 1.33 mg/ml (A-AgNPs), 1.98 mg/ml (E-AgNPs), 0.9 mg/ml (P-AgNPs) and 0.87 mg/ml (S-AgNPs) at 24 h (Fig. 3).

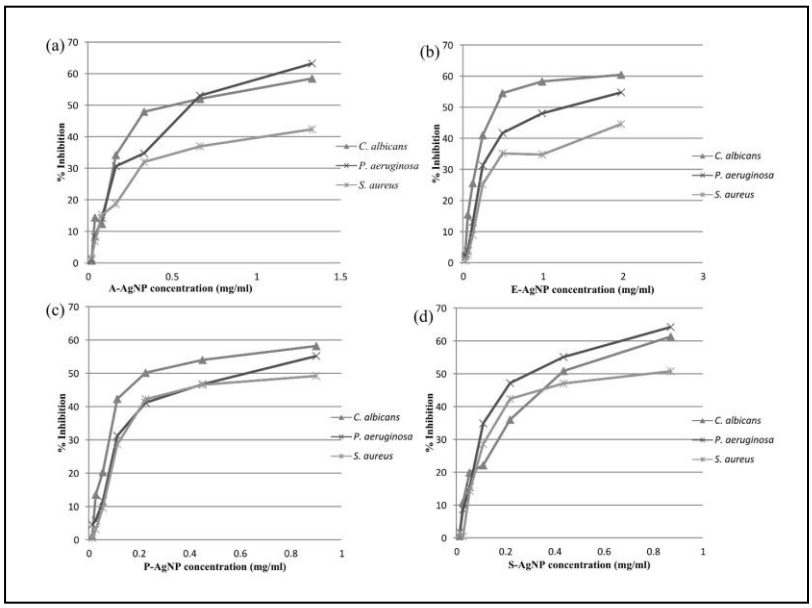


Fig. 3 Percentage inhibition of biofilms after 24 h treatment with the AgNPs produced by (a) *A. baumannii*, (b) *E. coli*, (c) *P. aeruginosa* and (d) *S. aureus*

Doubling dilutions of AgNPs were used to treat the 48 h old biofilms to determine 50% biofilm inhibitory concentration against the three bacterial biofilms tested. Biofilm inhibition was observed to be dose dependent. All four AgNPs showed more than 50% of biofilm inhibition against biofilms formed by *P. aeruginosa* and *C. albicans* while *S. aureus* biofilms showed 50% inhibition at this concentration only when treated with S-AgNPs (Table 1). With regard to *S. aureus* biofilm inhibition, A-AgNPs at 1.33 mg/ml was able to inhibit 42% of the biofilms, while highest AgNP concentration tested for E-AgNPs (1.98 mg/ml) were able to inhibit 45% of the *S. aureus* biofilms (Fig. 3). Further, 49% biofilm inhibition was seen when treated with 0.9 mg/ml, P-AgNPs. Fifty percent biofilm inhibition against *S. aureus* biofilms was achieved by treating the biofilms with 0.87 mg/ml AgNPs biosynthesized from the same organism.

Table 1: Concentrations of green AgNPs showing more than 50% inhibition of biofilms after 24 and 48 h treatments

Biosynthesized AgNPs	>50% Inhibitory concentrations of AgNPs (mg/ml)					
	C. albicans biofilms		*P. aeruginosa* biofilms		*S. aureus* biofilms	
	24 h treated	48 h treated	24 h treated	48 h treated	24 h treated	48 h treated
A-AgNPS	0.665	0.333	0.665	1.33	-	0.665
E-AgNPS	0.495	0.495	1.98	0.495	-	-
P-AgNPS	0.225	0.225	0.9	0.225	-	0.45
S-AgNPS	0.435	0.218	0.435	0.435	0.87	0.87

The highest biofilm inhibition at 24 hours when treated with A-AgNPs was observed against *P. aeruginosa* biofilm (63%) while treatment with E-AgNPs (60%) and S-AgNPs (61%) showed higher inhibition against *C. albicans* biofilms. *C. albicans* (58%) had stronger inhibition when treated with P-AgNPs while S-AgNPs were able to inhibit *P. aeruginosa* (64%), *C. albicans* (61%) biofilms. The biofilm inhibitory concentrations resulting in >50% biofilm inhibition by the four AgNPs after 24 h and 48 h of treatment against biofilms of *C. albicans, P. aeruginosa* and *S. aureus.* A-AgNP, E-AgNP, P-AgNP and S-AgNP are AgNPs biosynthesized using *A. baumannii, E. coli, P. aeruginosa* and *S. aureus* respectively. (Dashes indicate that the AgNPs did not give >50% biofilm inhibition at the tested concentration).

Electron microscopic imaging revealed that treatment of 48 h old biofilms with AgNPs for 24 h resulted in visible cell destruction leading to reduction in cell number, reduced extracellular matrix and damaged cells in all bacterial biofilms. Based on the crystal violet assay, S-AgNPs was the most effective against all microorganisms tested (Fig. 3). Fig. 2 shows the SEM images of S-AgNPs against *P. aeruginosa, S. aureus and C. albicans* biofilms after 24 h of treatment. AgNP treated cells (24 h) had altered cell morphology and disruption of cell membranes were also visible. Presence of silver nanoparticles was confirmed by the EDAX spectrum. Blebbing of the cell surface was seen in some cells which indicate the apoptosis process in these cells. Treated biofilms had less extracellular matrix when in contact with AgNPs for 24 h. After AgNP treatment of *C. albicans* biofilm, *Candida* cells showed rough outer cell wall with blebbing and markedly reduced presence of pseudo hyphae in AgNP treated specimens, while microcolonies were still present. The AgNPs were seen attached to the surface of the yeast cells.

Discussion

Bacterial biofilm formation is a challenge in treatment of infections due their intrinsic antimicrobial resistance. Thus, control of biofilm formation is critical in management of biofilm mediated infections. Silver nanoparticles have been reported to have multiple properties leading to antimicrobial activity and biofilm inhibition.

The nanoparticle size is a critical factor in determination of antimicrobial and antibiofilm properties[8]. According to Morones et al.[8], AgNPs less than 10 nm of size can directly contact with bacterial cells and enhance antimicrobial activity. The nanoparticles reported in this study

had an average particle size of <20 nm and had a spherical morphology. The smaller size of the nanoparticles can increase the antimicrobial potency due to the higher surface to volume ratio which facilitates greater interaction with the cell surface. Nanoparticles with small size have superior penetrating ability.[8]

The appearance, size, shape and particle distribution depend on surface plasmon resonance energy of AgNPs.[14] Further characterization of biosynthesized AgNPs using FT-IR spectroscopy indicated NH stretch vibrations present in the amide linkages of primary and secondary amines of proteins, presence of nitrocompounds from proteins or enzymes, C-H symmetrical stretch vibration of alkanes and -C-N- stretching vibrations confirming that the attached proteins in the biosynthesized AgNPs support stabilization of AgNPs.[15]

In the present study, silver nanoparticles synthesised by all four bacterial species demonstrated over 50% biofilm inhibition at a AgNP concentration between 1.98 mg/ml to 0.225 mg/ml (Fig. 3). The antibiofilm effectiveness of the AgNPs in the present study shows good promise when used at lower concentrations of approximately 1 mg/ml as seen in this study. Several studies have determined the effective biofiom inhibitory concentration of AgNPs. Martinez-Gutierrez et al.[9] demonstrated strong biofilm inhibition against *Pseudomonas* and *Acinetobacter* biofilms while weaker biofilm inhibitory activity was observed against MRSA and *Candida albicans* biofilms using high AgNP concentrations ranging from 500 – 1000 mg/ml. In contrast, a study by Barapatre et al.[14] using biosynthesized AgNPs (100 nm) had a minimum bactericidal concentration (MBC) of AgNPs between 16 µg/ml to 64 µg/ml against *S. aureus*, *E. coli* and *P. aeruginosa*. These differences may be due to the multiple factors contributing to the antimicrobial properties of the nanoparticles including the source of the AgNP, culture and biosynthesis parameters used, nanoparticle size and shape, surface charge, concentration, test organism and biofilm forming ability.

Biofilm formation is dependant on a number of factors and cell signalling through quorum sensing is one of the critical factors involved. AgNPs are involved in quorum quenching thereby diminishing the cell signalling as demonstrated by Chaudhari et al.[16] against *S. aureus* biofilms. Further, AgNPs may arrest the synthesis of exopolysaccharides (EPS) which is an essential pre-requirement for biofilm formation.[17] A study by Chandra et al.[2], demonstrated the three phases (early, intermediate and maturation) of biofilm formation of *C. albicans* using SEM imaging at different time points. The early phase of biofilm formation lacked detectable matrix, in the intermediate phase, cell density increased and formation of microcolonies was observed after 11 h while the mature biofilms were seen to have abundant matrix. The exopolysaccharides play an important role in protecting the biofilm from desiccation and antimicrobial agents.[18] Further, EPS helps in cell adhesion and facilitates cell to cell communication. EPS also has a role in cell organization and contributes to structural integrity of the biofilm. Therefore targeting the EPS can be a useful approach in biofilm control. In the present study and in studies by others, the treatment of AgNPs to bacterial biofilms induced several structural changes as observed in the SEM images. Reduction in the biofilm associated EPS was observed for all the four test organisms comparable to other reported studies indicating the arrest of biofilm EPS secretion. The attachment of AgNPs to the surface of microbial cells

was observed in this study using SEM and has also been reported by several groups.[17,19] Oza et al.[20] reported that the antibacterial effect of AgNPs may be due to penetration of released Ag ions in bcterial cell wall. AgNPs can disrupt the cell wall and rapidly detach the biofilms. Further, AgNPs can cause the formation of reactive oxygen species (ROS) which inhibits the antioxidant defense system and causes mechanical damage to the cell membrane.[21] The overall antibiofilm activity of AgNPs may therefore occur by several mechanisms.

In the present study, AgNPs biosynthesized from different bacterial species were able to inhibit the bacteial and *Candida* biofilms. An interesting observation was, that against the *S. aureus* biofilms, the most effective AgNP was the NPs synthesized by *S. aureus* itself. Among the Gram negative bacterial biofilms tested, S-AgNPs were also found to be strongly effective against *C. albicans* and *P. aeruginosa* biofilms compared to other AgNPs synthesized. Especially in *P. aeruginosa* biofilms treated with AgNPs, the morphology change was visible with increased roughness, as reported previously for *E. coli*[17], suggesting cell membrane damage by AgNPs. Comparable to previously published studies using planktonic cells, *S. aureus* biofilms were more resistant to AgNPs compared to Gram negative bacterial biofilms[11,22]. The structure of the cell wall can influence the adhesion and penetration of the nanomaterial into the cell. Electron microscopic studies suggest that the higher cell wall thickness of Gram positive bacteria compared to the Gram negative bacterial cell wall may act as a barrier to the penetration of the nanoparticles into the cell.[23] Thus Gram positive bacterial cells posess an intrinsic resistance to the antimicrobial activity of nanoparticles compared to Gram negative bacteria as seen in this study and studies published by others.[11,22,24]

Recently the focus on natural "green" methods of nanoparticle synthesis has been on the increase considering the low cost and environment friendly approach of biosynthesis compared to the chemical and physical methods of nanoparticle production. In this study, the focus was to compare different AgNPs biosynthesized by four bacterial species and to determine their antibiofilm potential. Using well defined culture and biosynthesis conditions in all experiments, it was possible to obtain AgNPs with reproducible properties which was confirmed by UV-visible spectroscopy, TEM, SEM and other methods. The exact mechanism of biofilm inhibition by green AgNPs is not fully understood.

Conclusion

The AgNPs synthesized by bacteria had significant antibiofilm activity after 24 h exposure against Gram positive, Gram negative and *Candida* biofilms at low concentrations displaying morphological changes and reduced biomass. The duration of AgNP exposure was important to obtain higher inhibitory effect. The results suggest the potential of green AgNPs as antibiofilm agents in therapeutics.

Abbreviations

AgNPs: Silver nanoparticles
EPS: Extracellular polysachcharides
MBC: Minimum bactericidal concentration

A-AgNPs: AgNPs by *A. baumannii*
E-AgNPs: AgNPs by *E. coli*
P-AgNPs: AgNPs by *P. aeruginosa*
S-AgNPs: AgNPs by *S. aureus*

Acknowledgement
The authors wish to thank the University of Sri Jayewardenepura, Sri Lanka for providing financial support (ASP/01/RE/MED/2016/42) and laboratory facilities.

References

1. Seddiki S, Boucherit-Otmani Z, Boucherit K, et al. Fungal infectivities of implanted catheters due to Candida sp. Biofilms formation and resistance. *J Mycol Med*. 2015; 25(2):130-135. *doi: https://doi.org/10.1016/j.mycmed.2015.03.003*
2. Chandra J, Kuhn DM, Mukherjee PK, et al. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J Bacteriol*. 2001; 183(18):5385-5394. *doi: http://dx.doi.org/10.1128/JB.183.18.5385-5394.2001*
3. Imamura Y, Chandra J, Mukherjee PK, et al. *Fusarium* and *Candida albicans* biofilms on soft contact lenses: model development, influence of lens type, and susceptibility to lens care solutions. *Antimicrob Agents. Chemother*. 2008; 52(1):171-182. *doi: http://dx.doi.org/10.1128/AAC.00387-07*
4. Peters G, Locci R, Pulverer G. Microbial colonization of prosthetic devices. II. Scanning electron microscopy of naturally infected intravenous catheters. *Zentralbl Bakteriol, Mikrobiol Hyg B.* 1981;173(5):293-299. *PMID:6792814*
5. Hoiby N, Bjarnsholt T, Givskov, et al. Antibiotic resistance of bacterial biofilms. *Int J Antimicrob agents.* 2010; 35(4):322-332. *doi: http://dx.doi.org/10.1016/j.ijantimicag.2009.12.011*
6. Goswami SR, Sahareen T, Singh M, et al. Role of biogenic silver nanoparticles in disruption of cell-cell adhesion in *Staphylococcus aureus* and *Escherichia coli* biofilm. *Ind Eng Chem Res*. 2015; 26:73-80. *doi: http://dx.doi.org/10.1016/j.jiec.2014.11.017*
7. Naik K, Kowshik M. Anti-quorum sensing activity of AgCl-TiO_2 nanoparticles with potential use as active food packaging material. *J Appl Microbiol*. 2014;117(4):972-983. *doi: https://doi.org/10.1111/jam.12589*
8. Morones JR, Elechiguerra JL, Camacho A, et al. The bactericidal effect of silver nanoparticles. *Nanotechnology*. 2005; 16(10):2346. *doi: http://dx.doi.org/10.1088/0957-4484/16/10/059*
9. Martinez-Gutierrez F, Boegli L, Agostinho A, et al. Anti-biofilm activity of silver nanoparticles against different microorganisms. *Biofouling*. 2013; 29(6):651-660. *doi: http://dx.doi.org/10.1080/08927014.2013.794225*
10. Jeevan P, Ramya K, Rena AE. Extracellular biosynthesis of silver nanoparticles by culture supernatant of *Pseudomonas aeruginosa*. *Ind J Biotechnol*. 2012; 11(1):72-76. *No doi*
11. Paul D, Sinha SN. Extracellular Synthesis of Silver Nanoparticles Using *Pseudomonas aeruginosa* KUPSB12 and Its Antibacterial Activity. *Jordan J Biol Sci.* 2014; 7(4):245-250. *doi: http://dx.doi.org/10.12816/0008246*
12. Singh R, Wagh P, Bellare J. Synthesis, optimization, and characterization of silver nanoparticles from *Acinetobacter calcoaceticus* and their enhanced antibacterial activity when combined with antibiotics. *International journal of nanomedicine.* 2013; 8:4277-4290. *doi: https://dx.doi.org/10.2147%2FIJN.S48913*

13. Stepanović S, Vuković D, Hola V, et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *Apmis*. 2007; 115(8):891-899. *doi: http://dx.doi.org/10.1111/j.1600-0463.2007.apm_630.x*
14. Barapatre A, Aadil KR, Jha H. Synergistic antibacterial and antibiofilm activity of silver nanoparticles biosynthesized by lignin-degrading fungus. *Bioresour Bioprocess*. 2016; 3(1):1. *doi: http://dx.doi.org/10.1186/s40643-016-0083-y*
15. Peiris MMK, Fernando SSN, Jayaweera PM, et al. Comparison of Antimicrobial Properties of Silver Nanoparticles Synthesized from Selected Bacteria. *Indian J Microbiol*. 2018; 58(3):301-311. *doi: 10.1007/s12088-018-0723-3*
16. Chaudhari PR, Masurkar SA, Shidore VB, et al. Effect of biosynthesized silver nanoparticles on *Staphylococcus aureus* biofilm quenching and prevention of biofilm formation. *Nano-Micro Lett*. 2012; 4(1):34-39. *doi: http://dx.doi.org/10.1007/BF03353689*
17. Ansari MA, Khan HM, Khan AA, et al. Antibiofilm efficacy of silver nanoparticles against biofilm of extended spectrum β-lactamase isolates of *Escherichia coli* and *Klebsiella pneumoniae*. *Appl Nanosci*. 2014; 4(7):859-868. *doi: http://dx.doi.org/10.1007/s13204-013-0266-1*
18. Flemming H-C, Wingender J, Szewzyk U, et al. Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol*. 2016; 14(9):563. *doi: http://dx.doi.org/10.1038/nrmicro.2016.94*
19. Lara HH, Romero-Urbina DG, Pierce C, et al. Effect of silver nanoparticles on Candida albicans biofilms: an ultrastructural study. *J nanobiotechnology*. 2015;13(1):91. *doi: http://dx.doi.org/10.1186/s12951-015-0147-8*
    Oza G, Pandey S, Shah R, et al. Extracellular fabrication of silver nanoparticles using *Pseudomonas aeruginosa* and its antimicrobial assay. *Pelagia Res Lib Adv Appl Sci Res*. 2012; 3:1776-1783. *No doi*
20. Liao J, Mo AC, Wu HK, et al. Antibacterial activity of silver-hydroxyapatite/titania nanoparticles on oral bacteria. In Key Engineering Materials. *Trans Tech Publ*: 2007: 299-302. *doi: http://dx.doi.org/10.4028/www.scientific.net/KEM.330-332.299*
21. Peiris MK, Gunasekara CP, Jayaweera PM, et al. Biosynthesized silver nanoparticles: are they effective antimicrobials? *Mem Inst Oswaldo Cruz.* 2017;112(8):537-543. *doi: http://dx.doi.org/10.1590/0074-02760170023*
22. Singh M, Mallick A, Banerjee M, et al. Loss of outer membrane integrity in Gram-negative bacteria by silver nanoparticles loaded with *Camellia sinensis* leaf phytochemicals: plausible mechanism of bacterial cell disintegration. *Bulletin of Materials Science*. 2016; 39(7):1871-1878. *doi: http://dx.doi.org/10.1007/s12034-016-1317-5*
23. Senarathna U, Fernando S, Gunasekara T, et al. Enhanced antibacterial activity of TiO_2 nanoparticle surface modified with *Garcinia zeylanica* extract. *Chem Cent J*. 2017; 11(1):1-7. *doi: http://dx.doi.org/10.1186/s13065-017-0236-x*

Research article

Characteristics of community acquired and hospital acquired methicillin resistant *Staphylococcus aureus* isolates in the National Hospital of Sri Lanka

WAMP Samaranayake[1], L Karunanayake[1], CGUA Patabendige[2]

Sri Lankan Journal of Infectious Diseases 2019 Vol.9 (1)24-31

DOI: http://dx.doi.org/10.4038/sljid.v9i1.8229

Abstract

Introduction and Objectives: Highly virulent community acquired methicillin resistant *Staphylococcus aureus* (MRSA) strains emerged recently causing infections in healthy young adults without predisposing factors. This descriptive cross-sectional study was conducted to compare socio-demography of patients and microbiology and molecular characteristics of Community acquired (CA) and Hospital acquired (HA) methicillin resistant *S. aureus* strains isolated at the National Hospital of Sri Lanka.

Methods and Results: Antimicrobial susceptibility test and Panton Valentine Leukocidine (PVL) gene detection was carried out on 100 MRSA isolates. CDC epidemiological criteria were used for differentiation of CA and HA MRSA. Of those 100 isolates, 21(21%) were CA-MRSA and 79(79%) were HA-MRSA. Patients did not show any significant difference in acquiring CA MRSA and HA MRSA in relation to their age, sex and gender except ethnicity. The majority of these isolates were from pus samples. CA-MRSA isolates were significantly more sensitive to ciprofloxacin, fusidic acid, tetracycline, cotrimoxazole, and gentamicin compared with HA-MRSA isolates ($p<0.001$). Inducible, constitutive clindamycin resistance ($p<0.001$) and multidrug resistant phenotypes were significantly higher ($p<0.001$) among patients with HA-MRSA infection. All isolates were susceptible to glycopeptides, rifampicin and linezolid. Mupirocin resistance was seen in 6% and all isolates came from patients who harboured HA-MRSA strains ($p<0.338$). The PVL gene ($P<0.001$) was present in 20 (95.2%) of CA-MRSA isolates.

Conclusion: This study highlights the importance of accurate differentiation of CA and HA MRSA using epidemiological, microbiological and molecular characteristics. Further, awareness of the existence of these types will optimise individual treatment strategies.

Key words: CA-MRSA, HA-MRSA, Antimicrobial resistance, Sri Lanka

[1]*Department of Bacteriology, Medical Research Institute, Colombo 08, Sri Lanka*
[2]*Department of Microbiology, National Hospital of Sri Lanka, Colombo, Sri Lanka*

Address for correspondence: Dr Manori Samaranayake, Unit 51,105 Bridge Road, Westmead, New South Wales, Australia Telephone: +61(02)0437208795

Email:manorisamaranayake1981@gmail.com *https://orcid.org/0000-0003-2459-9006*

Received 25 September 2018 and revised version accepted 8 February 2019

Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) is one of the major nosocomial pathogens in Sri Lanka that causes mild to life threatening infections. Prevalence of MRSA in Sri Lanka varies among the hospital settings from 47% to 62% while most are resistant to many antimicrobials tested.[1,2,3] In the late 1990s, a phenotypically and genotypically distinct highly virulent MRSA clone emerged as community-acquired/associated MRSA (CA-MRSA) causing skin and soft tissue infection, and severe haemorrhagic pneumonia in children and young adults without any predisposing conditions.[4] It usually carries smaller staphylococcal cassette chromosome mec (SCCmec) elements e.g. IV, V that do not contain other resistance genes and many clones spread independently worldwide. They produce Panton-Valentine Leukocidin toxin (PVL) which is responsible for both skin infection and severe haemorrhagic necrotizing pneumonia through tissue necrosis and abscess formation.[5] Many studies have shown significant association of PVL gene with CA-MRSA isolates compared with HA-MRSA isolates despite its controversial significance.[5,6] However, the prevalence of CA-MRSA varies markedly worldwide. Song *et al*. showed 25.5% prevalence of CA-MRSA in Asian countries with Sri Lanka demonstrating a higher prevalence of 38.8% in a multicentre surveillance study.[7] Local studies to assess the burden and characteristics of CA-MRSA and HA-MRSA infections in the country are lacking.

The purpose of this study was to compare the presence of PVL gene and antimicrobial susceptibility of CA and HA MRSA strains and to compare the socio-demographic features in patients with CA-MRSA and HA-MRSA infections in the National Hospital of Sri Lanka (NHSL).

Method

A descriptive cross-sectional study was conducted from November 2013 to March 2014 for statistically calculated 100 consecutive, non-repetitive MRSA isolates collected from the microbiology laboratory, National Hospital of Sri Lanka (NHSL).

HA-MRSA infection was defined as isolation of MRSA in a patient 48 hours after admission, with a history of hospitalization, surgery, dialysis, or residence in a long-term health care facility within the last one year prior to the culture date or who had an indwelling intravenous line, catheter or any other percutaneous medical device present at the time of isolation. Isolates with none of the above were classified as CA-MRSA.[8] An interviewer administered pre-piloted questionnaire was filled after written informed consent. Bed head tickets, clinic records and the guardian's histories were used in addition.

MRSA isolates were identified using standard protocols[9,10] in the microbiology laboratory, Medical Research Institute (MRI). Methicillin resistance was screened with cefoxitin 30µg disk and confirmed by PBP2a latex agglutination test (OXOID; DR0900) according to the manufacturer's protocol.[11] Antibiotic susceptibility was determined according to Clinical Laboratory Standard Institute (CLSI-guidelines 2013), for penicillin, rifampicin, cotrimoxazole, ciprofloxacin, gentamicin, tetracycline, linezolid and fusidic acid.[4] Inducible clindamycin resistance was identified by 'D-zone' tests with erythromycin (15µg) and clindamycin (2µg) disks.

Inducible and constitutive Macrolide-lincosamide-streptogramin B (MLSB) phenotypes were assessed by CLSI guidelines 2013.[11] Double-Disk diffusion testing method as described by Swenson *et al*, 2010 was used to detect high-level and low-level susceptibility with mupirocin 200µg and 5µg disk.[12] Glycopeptide susceptibility of MRSA was tested with vancomycin and teicoplanin (EzyMIC, Himedia) strips according to manufacturer's guidelines. Interpretations of susceptibility were done using CLSI 2013 guidelines.[11] A multi-drug resistant (MDR) isolate was defined as non-susceptibility to more than 3 antimicrobial classes and a pan drug resistant (PDR) isolate was defined as non-susceptibility to all antimicrobial agents.[12]

Conventional PCR was done to detect the PVL gene as described by Lina *et al*. (2009) using lukS PV(5-ATCATTAGGTAAAATGTCTGGACATGATCC A-3) and lukFPV(5-GCATCAA STGTATTGGATAGCAAAAGC-3) as primers.[13] *S. aureus* ATCC 25923 was used as the positive control[14] while *S. aureus* ATCC 25913 was used as the negative control.[15] DNA was extracted with the Wizard® Genomic DNA Purification Kit.[16] After amplification for 30 cycles (30s denaturation at 95 °C, 60s annealing at 55 °C and one minute extension at 72 °C), the PCR products were resolved by electrophoresis through 2% agarose gel. This was followed by ethidium bromide staining and analysis to visualise bands at 433bp. PCR was optimized to identify the best annealing temperature (55 °C) and primer concentration (0.3µM). Analytical sensitivity of the procedure was done for ten-fold serial dilutions of *S. aureus* (the positive control). Lower limit of detection was $3X10^4$ CFU/mL. Sensitivity was increased up to $3X10^3$ CFU/mL by increasing the number of cycles to 40. All hundred samples were subjected to the optimised PCR procedure with positive and negative strains included in each run.

Descriptive analysis was employed in investigating the distributions of variables between the HA and CA groups using SPSS16. Categorical variables between the two groups were compared by means of the chi-square test or Fisher's exact test. Range was used to assess the statistical dispersion of the data set. Statistical significance was assumed if P value was <0.05.

Results

One hundred MRSA isolates were tested in the study. The mean and median ages of the CA-MRSA group were 45 years and 57 years and that for HA-MRSA were 46.67 years and 56years respectively. The basic demographics of MRSA positive patients are shown in Table 1.

Table 1: Socio-demography of patients with CA-MRSA and HA-MRSA infections

	CA MRSA count	HA MRSA count	Odds ratio	95% Confidence Interval		P value
				Lower	Upper	
Female	**21**	**7**	0.724	0.257	2.040	0.541
Male	**58**	**14**				
Sinhalese	**14**	**70**	.257	.082	.806	0.020
*Non-Sinhalese	**7**	**9**				
< 45years	**9**	**34**	.993	.375	2.624	0.988
>45years	**12**	**45**				

*Non-Sinhalese: Tamil, Muslim and other ethnic groups

In this study, the prevalence of HA-MRSA was 79% (70.9%- 87.1%) and CA-MRSA was 21% (12.9% - 29.1%). The majority (92) were clinical samples and 8 were screening samples. Skin and soft tissue infections were the most common infection among all subjects. Blood and respiratory specimens had only HA-MRSA infections (Table 2).

Table 2: Type of samples

	CA-MRSA		HA-MRSA	
	No	**%**	**No**	**%**
Screening	3	37.5	5	62.5
Clinical	18	19.6	74	80.4
Blood stream infection	-		5	6.8
Respiratory tract infection	-		3	4.1
Skin/soft tissue infection/pus	16	88.9	61	82.4
Sterile fluid	1	5.6	5	6.8
Urinary tract infection	1	5.6	-	

Antibiotic susceptibility pattern is shown in Table 3. Resistance rates were significantly higher for fusidic acid, cotrimoxazole, tetracycline, ciprofloxacin, gentamicin, and clindamycin among isolates of HA-MRSA which showed the MDR phenotype.

All isolates were susceptible to rifampicin and linezolid but resistant to penicillin. The distribution of glycopeptide MIC values among the two groups differed. There were no glycopeptide intermediate or resistant *S. aureus* isolates. Prevalence of mupirocin resistance was 6%. All resistant isolates were in the HA-MRSA group. Four isolates showed high-level resistance and two isolates showed low-level resistance.

The proportion of PVL gene among HA-MRSA isolates was 3.8% whereas proportion of PVL among CA-MRSA isolates was 95.2 % ($p< 0.001$).

Table 3: Interpretation of antimicrobial susceptibility testing of the isolates

Antibiotic	Susceptibility result	HA MRSA *n*	CA MRSA *n*	P value
Fusidic acid 30µg	Resistant	**35**	**0**	<0.001
Cotrimoxazole	Resistant	**34**	**1**	<0.001
Tetracycline 30µg	Resistant	**56**	**1**	<0.001
Ciprofloxacin 5µg	Resistant	**61**	**1**	<0.001
Gentamicin 10µg	Resistant	46	3	<0.001
Inducible and constitutive clindamycin resistance (Erythromycin15µg+Clindamycin 2 µg)	Resistant	62	9	<0.001
Rifampicin 5µg	Resistant	**0**	**0**	NA
Linezolid 30µg	Resistant	**0**	**0**	NA
MDR (Multi drug resistance)	MDR	**57**	**0**	<0.001
XDR / PDR	XDR/PDR	**0**	**0**	NA
Vancomycin MIC	Range	0.5-2µg/ml	0.5-2µg/ml	NA
	MIC 50	1 µg/ml	1 µg/ml	
	MIC90	1.5 µg/ml	1.5 µg/ml	
Teicoplanin MIC	Range	0.25-3 µg/ml	0.25-1µg/ml	NA
	MIC 50	0.5 µg/ml	0.5 µg/ml	NA
	MIC90	1 µg/ml	0.5 µg/ml	

MDR -Multi drug resistant phenotype, PDR -Pan-drug resistance, XDR - Extreme drug resistance, MIC-Minimum inhibitory concentration, NA-Not applicable.

Discussion

The prevalence of CA-MRSA varies markedly worldwide. The NHSL is the largest tertiary care hospital in the country and caters to patients from all parts of the country. This study demonstrates that high proportions (21%) of isolates are CA-MRSA at NHSL. CA-MRSA categorization was done according to the patient's history which is based entirely on epidemiological information. However, the boundaries between HA-MRSA and CA-MRSA are becoming blurred due to the movements of patients and infections between hospitals and the community.[1]

The majority of the study population had skin and soft tissue infections. HA-MRSA was common in invasive samples such as blood and lower respiratory samples showing that HA-MRSA is prone to cause more invasive disease. None of the demographic factors such as age and gender (except ethnicity) were significant associates among the two groups which may be due to the small sample size. However, evidence suggests that CA-MRSA causes infection in healthy, predominantly young hosts who have no predisposing co-morbidities and in certain groups (ethnic groups, MSM, sport teams).[17,18,19] Clindamycin is used to treat serious infections caused by MRSA strains to suppress toxin production.[4] Constitutive and inducible clindamycin resistance was significant among HA-MRSA isolates in our study confirming global evidence.[2,3,15,17] CA-MRSA isolates in NHSL were significantly more susceptible to other antibiotics such as fusidic acid, cotrimoxazole, tetracycline, ciprofloxacin and gentamicin. The MDR phenotype was significantly higher in the HA-MRSA isolates than the CA-MRSA isolates. This highlights the importance of enforcing rational use of antimicrobials in the hospital setting.

The dissemination of MRSA has led to a tremendous increase in the use of glycopeptides worldwide. All our isolates were within the susceptible range of glycopeptide MIC values, similar to other local studies.[2] MIC50 shows how good an antimicrobial works intrinsically against a species while MIC90 reflects different resistance mechanisms. The vancomycin MIC's of the isolates suggest a drift towards antibiotic resistance. Teicoplanin MIC50, MIC90 and the range among CA-MRSA were less than that of HA-MRSA, reflecting the infrequent use of teicoplanin in the community setting. However, these data should be confirmed by large inter centre studies. There were five isolates which had vancomycin MIC of 2 μg/ml and seven isolates had teicoplanin MIC value ≥ 1.5μg/ml. We did not assess the clinical outcomes, hVISA strains (MIC 0.5–2 μg/ml) and vancomycin creep in our study. There are reports of poor clinical outcome and increased mortality in *S. aureus* infection with MIC's at the upper end of the susceptible range.[20, 21]

All isolates in our study showed susceptibility to linezolid and rifampicin, similar to other local studies.[2,3] Mupirocin is a topical agent which is used to treat skin infections and to eliminate nasal carriage of *S. aureus*. Usually high-level resistance is identified as an 'independent predictor' of decolonization failure while low-level resistant strains can recolonize very commonly.[22] In our study, prevalence of mupirocin-resistance was 6% which highlights the importance of ensuring restrictive use of mupirocin to prevent widespread resistance. Although double disk diffusion method has good sensitivity and specificity compared to broth dilution MIC, false negatives may occur rarely when there is a frame shift mutation in the mupA gene or silent mupA gene on the chromosome.[5]

Our study demonstrated significant presence of PVL gene among CA-MRSA ($p < 0.001$). Similar findings have been found globally.[6,17,21]

Conclusion

This study highlights the importance of collective use of clinical, microbiological and molecular tests for accurate differentiation of CA and HA infections. Antibiotic policy should be developed separately for the two groups of MRSA infections to optimise patient management.

Acknowledgement: We gratefully acknowledge the Director and staff of the Medical Research Institute, Colombo and Department of Microbiology at the National Hospital of Sri Lanka for their immense support.

Availability of data and materials: The datasets generated and/or analysed during the current study are not publicly available presently but are available from the corresponding author on request with no restriction.

Competing interest: There is no conflict of interest existing for any of the listed authors.

Funding: This research was funded by the Medical Research Institute, Colombo under the project no. 11/2013. There were no influences made on study design or methodology of collection, analysis, and interpretation of data and in writing the manuscript.

Ethics approval and consent to participate: The study was approved by the Ethics Review Committee, Medical Research Institute under the project no. 11/2013 and the Ethics Committee of the National Hospital of Sri Lanka under reference number AA/ETH/2013.

Informed written consent was taken from the patients before taking data for the questionnaire.

References

1. Fraimow HS, Tsigrelis C. Antimicrobial Resistance in the intensive care unit: mechanisms, epidemiology, and management of specific resistant pathogens. *J Crit Care Clin* 2011; 279:1:163-205 *doi: 10.1016/j.ccc.2010.11.002*.
2. Jayatilleke K, Bandara P. Antibiotic sensitivity pattern of *Staphylococcus aureus* in a tertiary care hospital of Sri Lanka. *Sri Lankan Journal of Infectious Diseases* 2012; 2(2):13-17. *doi: http://doi.org/10.4038/sljid.v2i2.4162*.
3. Patabendige CGUA, Chandrasiri NS, Karunanayake LI, et al. Antimicrobial resistance in resource-poor settings -Sri Lankan experience. Regional Health Forum 2011; 15:1. p.18-26. *No doi*
4. Mandell GL, Bennett JE, Dolin R. Principle and Practice of Infectious Disease.7th ed 2 United states: Churchill Livingstone Elsevier; 2010. p.355-2578.
5. Otto M. Community associated MRSA: a dangerous epidemic. *Future Microbiology* 2007; 2(5):457-459. *doi*: *https://doi.org/10.2217/17460913.2.5.457*
6. Nichol KA, Adam HJ, Mccracken M, et al. Comparison of community-associated and health care-associated methicillin-resistant Staphylococcus aureus in Canada: results of the CANWARD 2007-2009 study. *Diagn Microbiol Infect Dis* 2011; 69(3):320-5. *doi: 10.1016/j.diagmicrobio*.
7. Song JH, Hsueh PR, Chung DR, et al. Spread of methicillin-resistant *Staphylococcus aureus* between the community and the hospitals in Asian countries: an ANSORP study. *J Antimicrob Chemother* 2011; 66(5):10619 *doi: 10.1093/jac/dkr024.*
8. Minnesota Department of Health. Disease control newsletter; Community-Associated Methicillin-Resistant *Staphylococcus aureus i*n Minnesota 2004: 32(6):61-72. *No doi*
9. Barrow GI, Feltham RKA: Cowen and streel's: Manual for the identification of medical bacteria. 3rd ed. United Kingdom: Cambridge University Press;1993. PMC501641
10. MackieTJ, & McCartney JE. Practical Medical Microbiology.13th ed. United States: Churchill Livingstone Elsevier;1996. p.245-248.
11. Franklin RC, Jean BP, Jeff A, et al. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement. CLSI document M100-S23. Wayne, PA: Clinical and Laboratory Standards Institute; 2013.
12. Swenson JM, Wang B, Andrew ES et al. Multicentre study to determine disk diffusion and broth microdilution criteria for prediction of high and low-level Mupirocin resistance in *Staphylococcus aureus*. *J Clin Microbial* 2010; 48(7):2469–2475. *doi: 10.1128/JCM.00340-10.*
12. Magiorakos AP, Srinjvasa A, Carey B et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012;18(3):268-281 *doi: http://dx.doi.org/10.1111/j.1469-0691.2011.03570.x*
13. Lina G, Piemount Y, Gamot-Gamot F et al. Involvement of Panton-Valentine Leukocidin producing *Staphylococcus aureus* in primary skin infections and pneumonia *Clin Infect Dis* 1999; 29(5):1128–32. *doi: https://doi.org/10.1086/313461*.

14. Pérez JR, Tapia CO, Herazo CH, et al. Nasal carriage of Panton Valentine leucocidin positive methicillin resistant *Staphylococcus aureus* in healthy preschool children. *Rev. salud pública* 2011; 13(5):824-832. *doi: https://doi.org/10.1590/S0124-00642011000500011*.
15. McDanald RR, Antonishgn NA, Hansen T, et al. Development of a triplex real-time PCR assay for detection of Panton-Valentine Leukocidin toxin genes in clinical isolates of methicillin-resistant *Staphylococcus aureus. J Clin Microbial* 2005; 43(12):6147-6149. *doi: 10.1128/JCM.43.12.6147-6149.2005*.
16. Kumar R, Yadav BR, Dev K, et al. 2008. Protocol Online logo. A simple protocol for DNA extraction from *Staphylococcus aureus*: Protocol Online.http://www.protocolonline.org/prot/Protocol/A-Simple-Protocol-for-DNA-Extraction-from-Staphylococcus –Aureus-4999.html.Accessed on Oct 5, 2008.
17. Song JH, Hsueh PR, Chung DR, et al. Spread of methicillin-resistant *Staphylococcus aureus* between the community and the hospitals in Asian countries: an ANSORP study. *J Antimicrob Chemother* 2011; 66(5):1061-9. *doi: 10.1093/jac/dkr024.*
18. Bukharie HA. A review of community-acquired methicillin-resistant *Staphylococcus aureus* for primary care physicians. *J Family Community Med* 2010; 17(3);117–120. *doi: 10.4103/1319-1683.74320*
19. Otto M. MRSA virulence and spread. *Cell Microbiol* 2012; 14(10):1513–1521. *doi: https://doi.org/10.1111/j.1462-5822.2012.01832.x*
20. Deresinski S. Vancomycin hetero resistance and methicillin-resistant *Staphylococcus aureus*. *J Infect Dis* 2009; 199:605–9. *https://doi.org/10.1086/596630.*
21. Hiramatsu K, Cui L,Kuroda M et al. The emergence and evolution of methicillin resistant *Staphylococcus aureus*. *Trends Microbiol* 2001; 9:486-493. *https://doi.org/10.1016/S0966-842X(01)02175-8.*
22. Cookson BD. The emergence of mupirocin resistance: a challenge to infection control and antibiotic prescribing practice. *J Antimicrob Chemother* 1998; 41:11–18. doi: *http://dx.doi.org/10.1093/jac/41.1.11*

Research article

Baseline titres of O, H and AH agglutinins to *Salmonella* Typhi and Paratyphi A in blood donors in Sri Lanka.

SC Illapperuma[1], EM Corea[2], SB Agampodi[3]

Sri Lankan Journal of Infectious Diseases 2019 Vol.9(1):32-41

DOI: http://dx.doi.org/10.4038/sljid.v9i1.8230

Abstract

Background: Sri Lanka is considered as an endemic country for enteric fever. Due to difficulties in performing blood cultures, the Widal test is still commonly carried out for the diagnosis of enteric fever. However, there are no published data on current baseline Widal titres in the country. This study was carried out to determine the baseline titres of O, H and AH agglutinins among the Sri Lankan population.

Method: Five hundred and one (501) serum samples of blood donors from 31 blood banks in Sri Lanka were collected during 2012 and 2013 and were screened for *Salmonella* O, H and AH agglutinins using the Widal tube test. A titre of 20 and above was considered positive. Age and gender of the study participants were recorded.

Results: Of the 501 sera tested, 58% were positive for at least one of the O, H and AH agglutinins. *Salmonella* O, H and AH agglutinins were positive in 46.1%, 26.5% and 8.4% of the study population respectively. Of the study population, 97.5% had O, H and AH agglutinin titres less than or equal to 80, 160 and 80 respectively. A significantly higher percentage of females (H-36.7%, p=0.019; AH-15.3%, p=0.15) were positive for H and AH agglutinins than males (H-24.9%; AH-7.4%). The baseline titre of AH agglutinins was higher in females (160) than males or the total population (80). Highest test positivity (40%) was seen among the 31-40 year age group. A significant number of donors below 20 were negative for *Salmonella* O agglutinins (p=0.024).

Conclusion: We recommend baseline titres of 80, 160 and 80 for *Salmonella* O, H and AH agglutinins respectively to be used in Sri Lankan settings. As there is a variation in baseline titre with age and gender it is necessary to consider both when interpreting Widal test results.

***Keywords**: Widal test, Baseline values, Enteric fever, Typhoid, Paratyphoid, Sri Lanka*

---

[1]*Department of Microbiology, Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka, Saliyapura, Anuradhapura, Sri Lanka,*
[2]*Department of Microbiology, Faculty of Medicine, University of Colombo,*
[3]*Department of Community Medicine, Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka, Saliyapura, Anuradhapura, Sri Lanka*

Address for correspondence: S.C Illapperuma, Department of Microbiology, Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka, Saliyapura, Anuradhapura, Sri Lanka, Telephone: +94774120548

E mail: scillapperuma@gmail.com *https://orcid.org/ 0000-0002-8814-8530*

Received 5 October 2018 and accepted 25 February 2019

Background

Enteric fever includes both typhoid and paratyphoid fevers and is a global health problem. Typhoid fever is caused by *Salmonella* Typhi whilst the causative organism of paratyphoid fever is *Salmonella* Paratyphi A. The global estimate for typhoid fever in year 2000 was 21,650,974 with 216,510 deaths. In the same year, the estimated number of paratyphoid fever cases was 5,412,744.[1] By 2010, there was an increase in the incidence of typhoid fever with the estimated number being about 26.9 million with around 1% of global deaths attributed to this disease.[2]

Enteric fever is typically a disease associated with poverty and poor sanitation, hence commonest in low and middle income countries (LMIC). Disease transmission is via the faecal-oral route and contamination of food and water with infected faeces facilitates transmission of these diseases. With increasing global incidence and the development of multi-drug resistance to commonly used antibiotics, enteric fever is among the top public health problems in many LMICs, including Sri Lanka. However, basic information on enteric fever from some endemic countries is missing due to lack of recent research.

In order to arrive at a definitive diagnosis, isolation of either *S.* Typhi or *S.* Paratyphi A by culture of blood, bone marrow, stool, urine, rose spots or intestinal secretions is necessary.[3] Of these, bone marrow culture is considered to be the most reliable[4], but its invasive nature has limited its use.[5] Blood culture is considered the next best test in diagnosing enteric fever. However, its use is compromised in LMICs due to empirical antibiotic usage, cost, lack of laboratory facilities and time taken to obtain results.[5,6] Thus, the Widal test, based on the demonstration of antibodies against the H (flagellar) and O (somatic) antigens of *S.* Typhi and *S.* Paratyphi A in the serum of patients, is still commonly used for diagnosis of enteric fever in many countries, including Sri Lanka. However, demonstrating a four-fold rise in titre in paired acute and convalescent sera, as recommended, is not commonly practiced and the antibody titre in a single acute sample of serum is often used to decide on the clinical management of patients.

For the correct interpretation of a Widal test on a single sample of serum, it is important to know the baseline antibody titres in the normal population in a particular geographical area.[6,7] This is necessary as a significant number of healthy persons may also carry antibodies to *S.* Typhi and *S.* Paratyphi A.[6,8,9] In an endemic area, there is often a high background titre in the general population due to previous clinical or subclinical infection with *S.* Typhi or *S.* Paratyphi or previous vaccination for typhoid. Previous infection caused by non typhoidal *Salmonella* with O antigen type 9 or 12[10], *Salmonella* Enteriditis[4], other Enterobacteriaceae and even *Burkholderia pseudomallei*[11] and malaria[10] may lead to the development of Widal agglutinins. Lack of data on baseline *Salmonella* agglutinin titres in endemic populations may therefore lead to incorrect interpretation of the Widal test. At present, no literature is available on current baseline agglutinin titres of *S.* Typhi and *S.* Paratyphi in Sri Lanka. The purpose of this study was to determine the baseline agglutinin titres of *S.* Typhi and *S.* Paratyphi A in the healthy population in Sri Lanka.

Methods

The study population was healthy blood donors from Sri Lanka. The study sample included 501 serum samples obtained from donors from 31 of the 96 blood banks in Sri Lanka during 2012/2013 (Figure 1).

Fig. 1: Map of Sri Lanka showing locations of blood banks enrolled in the study and number of blood samples tested for each site.

Serum was separated and stored at -20 °C until further testing. Haemolysed as well as samples with inadequate sera were excluded from the study. Anonymized data on age and sex of blood donors was collected, and therefore the requirement for informed consent was waived by the Ethics Review Committee, Faculty of Medicine, University of Peradeniya, Sri Lanka.

Standard *Salmonella* Typhi O, *Salmonella* Typhi H and *Salmonella* Paratyphi AH antigens (Biotech Ltd, Suffolk, UK) were used to perform the tube Widal test. The antigens were validated using relevant antisera with known titres (Murex Biotech Ltd, Dartford, UK).

Tube agglutination tests were carried out according to the manufacturer's instructions. Two fold dilutions, from 1:20 to 1:1280, of each of the 501 serum samples was prepared as follows. Three sets of 8 Kahn tubes were used for each serum sample in order to test for agglutinins to each of the antigens *Salmonella* O, *Salmonella* H and *Salmonella* AH. To tube one 1.9ml of 0.85% saline was dispensed whilst1.0ml of 0.85% saline was dispensed into the other seven tubes. Thereafter, 0.1ml of undiluted patient's serum was pipetted into tube 1 and mixed thoroughly by vortexing.

Next, 1.0ml from tube 1 was pipetted into the second tube and mixed thoroughly. Similarly, doubling dilutions were continued through to the sixth tube. Serum was not added to the seventh tube which acted as the control tube. One drop of the appropriate undiluted antigen suspension was added to each tube. The tubes were vortexed and incubated in a water bath at 50 °C. The tubes containing *Salmonella* O antigen were incubated for 4 hours and the tubes with *Salmonella* H and AH antigens were incubated for 2 hours. The tubes were covered with aluminum foil, left overnight in a refrigerator, and examined for agglutination after 24 hours.

Large flakes of agglutination, visible to the naked eye, in the tubes with *Salmonella* H and AH antigens and small granules of agglutination, visible with a magnifying glass, in the tubes with *Salmonella* O antigen were considered as giving positive results after ensuing that the control tubes did not show auto-agglutination. The reciprocal value of the highest dilution at which agglutination was visible was taken as the end point titre. Any titre above 20 was taken as a positive test according to the manufacturer's instructions.

The percentage of positive sera in each blood bank was mapped. The percentage of the study population positive for each agglutinin was determined. The baseline titres of O, H and AH agglutinins were determined by finding the titre of each agglutinin present in 97.5 percent of the study population. Distribution of positivity for any agglutinin at any titre and test positivity for O, H and AH agglutinin by sex and age was determined and baseline titres for males and females was determined. A p value of < 0.05 in the Chi square test was considered significant.

Results

Of the 501 participants in the study, data on age were available from 459 individuals. Age range of the study population was 18 to 72 years, with the majority being in the 21-30 year age group (43.3%). Only 0.4% of blood donors were above 60 years of age. A majority in the study population were males (78%).

Distribution of positive serum samples

Positivity of any agglutinin, at any titre, is shown in Table 1. Of the 501 samples tested, 57.9% (290/501) showed titres of ≥ 20 for one or more of the three agglutinins O, H or AH (Table 1). A titre ≥ 40 for one or more agglutinins was shown by 187 of the 501 sera (37.3%).

Table 1. Distribution of positive sera for one or more of the three agglutinins O, H or AH

Widal status	N	%
Positive	290	57.9
Negative	211	42.1
Total	501	100

Distribution of agglutinin titres

Of the study population, 231/501 (46.1%) had O agglutinin titres of ≥ 20. Among them, 230 (99.6%) had titres between 20 and 80. Only one had a titre of 160 (Table 2.). Of the study population 133/501 (26.5%) had H agglutinin titres of ≥ 20. Among them, 126 (94.7%) had titres between 20 and160, 6 (4.5%) had a titre of 320 and only one had a titre of 640 (Table 3). Of the study population, 42/501 (8.4%) had AH agglutinin titres ≥ 20. Among them, 40 (95.2%) had titres between 20 and 160. Only two had a titre of 320 (Table 4).

Table 2. Distribution of O agglutinin titres among 501 blood donors from Sri Lanka

Highest Titre	N	%	Cumulative %
0	270	53.9	53.9
20	105	21	74.9
40	79	15.8	90.6
80	46	9.2	99.8
160	1	0.2	100
Total	501	100	

Table 3. Distribution of H agglutinin titres among 501 blood donors from Sri Lanka

Highest titre	n	%	Cumulative %
0	368	73.5	73.5
20	46	9.2	82.6
40	47	9.4	92
80	19	3.8	95.8
160	14	2.8	98.6
320	6	1.2	99.8
640	1	0.2	100
Total	501	100	

Table 4. Distribution of AH agglutinin titres among 501 blood donors from Sri Lanka.

Highest titre	n	%	Cumulative %
0	459	91.6	91.6
20	16	3.2	94.8
40	10	2	96.8
80	7	1.4	98.2
160	7	1.4	99.6
320	2	0.4	100
Total	501	100	

Table 5. Summary of antibody titres with percentiles.

Percentiles	O highest titre	H highest titre	AH highest titre
50	0	0	0
90	40	40	0
95	80	80	40
97.5	80	160	80

Percentile distribution of baseline antibody titres in sera of blood donors.

For all three antigens, the agglutinin titre at the 50th centile was zero. At the 97.5 percentile, the titre of O, H and AH agglutinins in the study population was less than or equal to 80, 160 and 80 respectively (Table 5).

Table 6: Distribution of titres of *Salmonella* O, H and AH agglutinins by sex

Agglutinin		Male		Female		Pearson Chi-Square Tests	
		n	%	n	%	Chi-square	P
O	Negative	207	56.60	47	48.00	2.307	0.129
	Positive	159	43.40	51	52.00		
H	Negative	275	75.10	62	63.30	5.48	.019*
	Positive	91	24.90	36	36.70		
AH	Negative	339	92.60	83	84.70	5.903	.015*
	Positive	27	7.40	15	15.30		

Details on the gender of blood donor were available in 464 samples. Test positivity (titre ≥ 20) of these sera for O, H, and AH agglutinins at any titre, distributed by gender, is shown in Table 6.

The rate of H and AH agglutinin positivity was significantly higher in females. This gender difference was not seen for O agglutinins (Table 6).

Percentile distribution of baseline titres by sex

The O and H agglutinin titres at the 97.5 percentile were equal in males and females and similar to the baseline titre of the study population. However, the titre of AH agglutinins in females was 160, which is greater than that in males and in the study population (80) (Table 7).

Table 7. Baseline titres of O, H and AH agglutinins

Percentiles	O highest titre		H highest titre		AH highest titre	
	Male	Female	Male	Female	Male	Female
50	40	20	0	0	0	0
90	40	80	40	80	0	20
95	80	80	80	160	40	80
97.5	80	80	160	160	80	160

Distribution of titres of *Salmonella* O, H and AH agglutinins by age

Age distribution of the 459 sera samples is shown in Table 8. Percentage distribution of positives by age for all agglutinins was lower in the population below 20 years. The highest positivity rate was seen in the 31-40 year age group (40.2%).

Table 8. Percentage distribution of positives by age for any one or more of the three agglutinins O, H or AH

	Negative		Positive	
Age	**N**	**%**	**N**	**%**
<20	7	3.6	6	2.3
21-30	95	49.2	88	33.1
31-40	50	25.9	107	40.2
41-50	31	16.1	43	16.2
51-60	9	4.7	20	7.5
>60	1	0.5	2	0.7
Total	193	100	266	100

Test positivity of 459 serum samples for O, H, and AH agglutinins distributed by age is shown in Tables 8 and 9. Positivity for O agglutinins is significantly low below the age of 20 years (Chi square = 5.062 p=0.024). It doubles between 21-30 years of age and then remains static. Positivity for H agglutinins is low below the age of 20 and rises with age. However, the difference between test positivity for H agglutinins among the age group below and above 20 years was not statistically significant (Chi square = 3.569 p=0.058). Widal positivity for AH agglutinins was low (8.38%). Positivity for AH agglutinins is low below the age of 40 and then doubles between 41-50 years of age. However, the difference between positivity for AH agglutinins in the age group below 20 years and above 21 years was not statistically significant (p=0.786 with Yates correction) (Table 9).

Table 9. Distribution of test positivity of O, H and AH agglutinins by age

Age group	O agglutinin				H agglutinin				AH agglutinin			
	Negative		Positive		Negative		Positive		Negative		Positive	
	n	%	n	%	n	%	n	%	n	%	n	%
<20	17	77.30	5	22.70	20	90.90	2	9.10	21	95.50	1	4.50
21-30	108	54.30	91	45.70	159	79.90	40	20.10	187	94.00	12	6.00
31-40	74	50.30	73	49.70	97	66.00	50	34.00	135	91.80	12	8.20
41-50	38	58.50	27	41.50	42	64.60	23	35.40	52	80.00	13	20.00
>50	15	57.70	11	42.30	16	61.50	10	38.50	24	92.30	2	7.70

Discussion

In this study, blood bank donors were chosen to represent the healthy Sri Lankan population. Blood donors are often used as representatives when reference ranges are calculated or assay methods validated because they are considered as healthy individuals in a population.[12] Many workers have used blood bank donors as representing the general population in studies on baseline titres of *Salmonella* agglutinins.[12,18] The blood banks in this study covered all Districts and Provinces of Sri Lanka, except for the Mullaitivu District which does not have a blood bank (Figure 1).

In the present study, the seroprevalence of one or more of *Salmonella* O, H, and AH agglutinins among blood donors was 57.8%, which shows that a large number of individuals in Sri Lanka are positive for antibodies to *Salmonella* antigens. This seropositivity rate is higher than that found in Kenya (21.25%)[12] and North Kerala (25.2%)[16], but much lower than that found in studies done in Maharashtra and Hubli-Dharwad[17,18], probably reflecting exposure rates in each population. The high seropositivity seen in Maharashtra[17] was also hypothesized to be due to exposure to cross reacting bacteria such as *Citrobacter freundii,* a harmless gut bacterium which share antigenic properties with *Salmonella* serotypes.

The seropositivity rate of 37.3% at a titre ≥ 40 found in the present island-wide study is higher than the 12.7% reported in a previous study done in Sri Lanka in 1965 which was limited to the Western Province where the starting dilution was 1:50.[8] The seroprevalence at a titre ≥ 40, of O, H and AH agglutinins found in the present study (O=24.75%, H=17.37%, AH=5.1%) is also considerably higher than the corresponding percentages in a previous study conducted in the Western Province.[8] This higher rate is maintained, even if we compare only the results for Colombo (O=25%, H=25%, AH=12.5%) with that found in 1965 in the Western Province (1.8%, 10.6% and 2.4%).[8] Further, the titres for Jaffna in the present study (O= 37.5%, H=25%, AH=6%) are higher than those found in Jaffna in 2012/2013 (7%, 9% and 0%).[19] This may be due to differences in methodologies, antigen kits and starting dilutions used in the different studies (1/20, 1/50 and 1/30 respectively). The higher positivity rate in Colombo in this study, compared to 1965 may also be explained as being caused by an increase in cross-reacting antibodies in the population of the Western Province. These cross reacting antibodies may have arisen due to increased prevalence of infections such as dengue[20], leptospirosis[21] or food poisoning due to *Salmonella* Enteritidis[22], as these organisms share antigens with typhoidal

Salmonella serotypes[23]. Such cross reacting antibodies are usually found at low titres[24] as seen in this study. Similar to Gunjal *et al*. (2013)[17] and Bijapur *et al.* (2014)[16], the seropositivity rate for O agglutinins was higher than that for H and AH agglutinins. This may be because of constant exposure to bacteria in the environment which share O antigens with enteric fever causing *Salmonellae.*

This high background level of test positivity makes it even more important to determine the baseline cut off titres for the Sri Lankan population before performing the Widal test to diagnose acute enteric fever. In this study, 97.5% had O, H, and AH agglutinin titres of less than or equal to 80, 160 and 80 respectively. The highest titre found in 97.5% of the population is considered as the background titre for that particular geographical area. Therefore, these titres can be considered as baseline titres of O, H and AH agglutinins in Sri Lanka and titres above these can be considered as indicators of acute infection.

The baseline Widal titres obtained in the present study are consistent with those found in other studies conducted in endemic areas such as India, Kenya and Nigeria.[6,16,17,18,12,25] These baseline titres are also consistent with a study done in Sri Lanka by Thevanesam (1992)[26] where the diagnostic cut off titres for typhoid fever were ≥ 120 for O agglutinin and ≥ 120 for H agglutinin respectively. However, they contrast with a study by Gnanakarunyan *et al* (2012)[19] in Jaffna, where the cut off titres were 480 and 60. This may be due to differences in study populations and source of SAT antigens, test methodology and starting serum dilutions.

The percentage of females with O agglutinins was similar to that in males but H and AH agglutinin positivity was significantly higher in females. This contrasts with a study done in Nigeria[25] where a higher positivity in males was seen, with males showing 39%, 41% and 51% positivity to O, H and AH agglutinins versus 10.7%, 29.5% and 17% in females. This shows that males and females in Sri Lanka are equally exposed to bacteria with antigens that cross react with *Salmonella.*

Exposure appears to be maximum in the 31-40 year age group as the highest positivity rates were seen in this group. O agglutinin positivity rates remain almost the same with increasing age after 20 years while H positivity rates gradually increase. O agglutinins are short lived and reflect current exposure to cross reacting bacteria. They also do not show an anamnestic reaction.[10,13] Therefore all age groups should have similar O agglutinin positivity. The findings of the present study agree with this. In contrast, H and AH agglutinins persist for longer after exposure and show an anamnestic reaction to re-exposure[13] and positivity increases with age. The findings of the present study are in accordance with this as well.

Our results are similar to the study conducted in 1965 by Velaudapillai and Singho[8] where an increase in H agglutinin positivity was seen with age in the non-vaccinated normal population. This increasing trend of seropositivity with age is important when interpreting a Widal test result. Widal positivity for any one or more of the three agglutinins O, H or AH is low (2.3%) under 20 years of age. However, the difference between those under 20 years and those over 20 years was significant only for O agglutinin titres. The low positivity rate in this age group reflects less exposure to bacteria with cross reacting antigens. A consequence of this result is that baseline titres in the paediatric population are different (lower) than for adults and that cut off titres should

be adjusted to reflect this difference. Further studies are needed to elucidate the cut off titres in the paediatric age group.

Limitations

The baseline agglutinin titres that are reported in this study may not truly reflect that of the general population due to the smaller percentage of females (21.1%) in the study population and the lack of samples from persons aged <20 years (4.8%) and >50 years (5.6%). The employment of different antigen kits, some commercial and some in-house, and different methodologies in various laboratories in Sri Lanka make comparisons between baseline titres derived from different studies difficult.

Conclusions

Although the background prevalence of *Salmonella* O, H and AH agglutinins in the healthy population in Sri Lanka is high, the titres were seen to be low, with baseline titres being 80, 160 and 80 for *S.* Typhi O, *S.* Typhi H and *S.* Paratyphi AH agglutinins respectively. Therefore, titres above these values can be used to diagnose enteric fever. It is important to consider sex and age when interpreting a positive test. It would be advisable to regularly update the baseline titre values for different geographical areas.

Acknowledgement: HETC project of the Ministry of Higher Education, Sri Lanka for providing financial assistance through the grant: RUSL/O-MAS/N6

Prof D.J Weilgama, Merit professor in Parasitology, for his immense support in correcting the manuscript. Dr. M.G.R.S.S. Gunathilake, Lecturer, Department of Microbiology, Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka for the generous help extended in preparing the final manuscript. Mr. Sirithilak Gamage, Senior Technical Officer and all other staff of Department of Microbiology, Faculty of Medicine, University of Colombo for all their technical support provided throughout the study

References

1. Crump JA, Luby SP, Mintz ED. The global burden of typhoid fever. *Bull World Health Organ*. 2004; 82(5):346–353. *No doi* PMID: 15298225 PMCID: PMC2622843
2. Buckle GC, Walker CL, Black RE. Typhoid fever and paratyphoid fever: Systematic review to estimate global morbidity and mortality for 2010. *J Glob Health*. 2012; 2(1):010401. *doi:10.7189/jogh.02.010401*
3. Pegues DA, Ohl ME MS. Salmonella species, including Salmonella Typhi. In: Mandell GL, Bennett JE and Dolin R (eds). Principals and practice of infectious diseases. 7th edition, New York: Churchill Livingstone, 2005, pp. 2636–50.
4. Chart H. Salmonella- Food Poisoning: enteric fever. In: David Greenwood, Richard Slack Michael Barer, Will Irving (eds). Medical Microbiology. A Guide to Microbial Infections: Pathogenesis, Immunity, Laboratory Investigation and Control. 18th edition, Churchill Livingstone: Elsevier, 2012: pp. 265–74.
5. Bhutta Z.A andMansurali N. Rapid serologic diagnosis of pediatric typhoid fever in an endemic area: A prospective comparative evaluation of two dot-enzyme immunoassays and the Widal test. *Am. J. Trop. Med. Hyg*. 1999; 61:654–657.

doi: 10.4269/ajtmh.1999.61.654
6. Pal S, Prakash R, Juyal D, et al. The baseline Widal titre among the healthy individuals of the hilly areas in the garhwal region of uttarakhand, India. *J Clin Diagn Res*. 2012; 7(3):437–440. *doi:10.7860/JCDR/2013/4889.2793*
7. Cheesebrough M. District Laboratory Practice in Tropical Countries (2nd ed). Cambridge University Press., 2006.
8. Velaudapillai T, Singho LAA. A Study of enteric fever agglutinins in Ceylon. *Ceylon J of Med Sciences*, 1965; XIV. *No doi*
9. Zorgani A, Ziglam H. Typhoid fever: Misuse of Widal test in Libya. *J Infect Dev Ctries* 2014; 8:680–7. *doi:10.3855/jidc.3700*
10. Olopoenia LA, King AL. Widal agglutination test - 100 years later: still plagued by controversy. *Postgrad Med J*. 2000; 76(892):80–84. *doi:10.1136/pmj.76.892.80*
11. Valsalan R, Shubha S, Mukhopadhyay C et al. False-positive Widal in melioidosis. *Indian J Med Sci* 2009;63. *doi:10.4103/0019-5359.57647*
12. Ezzi MS, Anzala O, Maritim MC, Bhatt KM. Distribution of *Salmonella* Typhi antibodies in the sera of healthy blood donors at Kenyatta National Hospital. *Online J Med Med Sci Res* 2014; 3(3):19-23. *No doi*
13. Pokharel P, Rai SK, Karki G et al. Study of enteric fever and antibiogram of Salmonella isolates at a teaching hospital in Kathmandu Valley. *Nepal Med Coll J*. 2009; 11:176–8. *No doi PMID: 20334064*
14. Perera J, Perera R NH. Determination of standard agglutination test (SAT) titres in a normal population. *Proc. Sri Lanka Med. Assoc. 108th Anniv. Acad. Sess*., 1995.
15. Bahadur AK, Peerapur BV. Baseline titre of Widal amongst healthy blood donors in Raichur, Karnataka. *J Krishna Institute of Med Sciences University*. 2013; 2(2):30–36. No doi
16. Bijapur GAM, Kakkeri SR, Raysa NP, Usman SM. A study to determine significant titre-values of Widal test in the diagnosis of enteric fever for a population of north Kerala, India. *Al Ameen J Med Sci*. 2014; 7(1):71–77. *No doi*
17. Gunjal SP, Gunjal PN, Patil NK et al. Determination of baseline Widal titres amongst apparently healthy blood donors in Ahmednagar, Maharashtra, India. *J Clin Diagn Res* 2013; 7:2709–11. *doi:10.7860/JCDR/2013/6252.3738.*
18. Madhusudhan NS and Manjunath AH . Determination of baseline Widal titre among healthy population. *Int J Biomed Res* 2013;3:437. *doi:10.7439/ijbr.v3i12.843.*
19. Gnanakarunyan T, Thevanesam V and Gamage TM. Determination of diagnostic cut off values for the Widal test in the Jaffna district. *Sri Lanka Soc Microbiol* 2014:20. *No doi*
20. Kanakaratne N, Wahala WMPB, Messer WB et al. Severe dengue epidemics in Sri Lanka, 2003–2006. *Emerg Infect Dis* 2009; 15:192–9. *doi:10.3201/eid1502.080926*.
21. Agampodi SB, Peacock SJ, Thevanesam V et al. Leptospirosis outbreak in Sri Lanka in 2008: lessons for assessing the global burden of disease. *Am J Trop Med Hyg* 2011; 85:471–8. *doi:10.4269/ajtmh.2011.11-0276.*
22. Mubarak F, Chandrasiri P. A case series of nontyphoidal Salmonella bacteraemia- some food for thought! *Sri Lankan J Infect Dis* 2013; 3:37–42. *doi: http://doi.org/10.4038/sljid.v3i2.4711*
23. Ohanu ME, Mbah AU, Okonkwo PO, et al. Interference by malaria in the diagnosis of typhoid using Widal test alone. *West Afr J Med* 2003; 22:250–2. *doi:10.4314/wajm.v22i3.27961*.
24. Parry CM, Hoa NT, Diep TS, et al. Value of a single-tube widal test in diagnosis of typhoid fever in Vietnam. *J Clin Microbiol*. 1999;37(9):2882–2886. *no doi*
PMID: 10449469 PMCID: PMC85403
25. Ibekwe AC, Okonko IO, Onunkwo AU, et al. Baseline Salmonella agglutinin titres in apparently healthy freshmen in Awka, South Eastern, Nigeria. *Sci Res Essays* 2008; 3:425–30. *no doi*
26. V. Thevanesam. An evaluation of the SAT in the diagnosis of typhoid. *Ceylon Med J* 1992; 37:48–51. *no doi PMID: 1423751*

Research article

A preliminary study of *mecA* gene expression and methicillin resistance in staphylococci isolated from the human oral cavity

WMDA Wijesundara[1], RGSC Rajapakse[1],
JAMA Jayatilake[2], JAMS Jayatilake[3]

Sri Lankan Journal of Infectious Diseases 2019 Vol. 9(1):42-48

DOI: http://dx.doi.org/10.4038/sljid.v9i1.8232

Abstract

Introduction: Staphylococci are common human commensals that acquire methicillin resistance via the *mecA* gene. Methicillin resistance in staphylococci from various clinical sources has been assessed using cefoxitin disc diffusion test (CDDT) and PCR detection of the *mecA* gene. However, oral staphylococci have been studied less frequently compared with other clinical sources. There are no previous studies on methicillin resistance in oral staphylococci in Sri Lanka.

Objective: This study aimed to demonstrate methicillin resistance in staphylococci isolated from the human oral cavity using CDDT and PCR detection of *mecA* gene.

Materials and methods: Twenty-five oral isolates of staphylococci were selected after confirming their identity using colony morphology, Gram stain, catalase test, and the coagulase test. Further authentication of identity was obtained using amplification of the 16S rRNA gene. Methicillin resistance was demonstrated using CDDT and PCR detection of the *mecA* gene.

Results: There were 7 (28%) isolates of coagulase positive (presumed *S. aureus*) and 18 (72%) of coagulase negative staphylococci (CoNS). All the coagulase positive isolates were methicillin sensitive. Within the 18 CoNS, 2 (11%) were methicillin resistant and were found to carry the *mecA* gene using PCR.

Conclusion: Coagulase positive and negative staphylococci with or without methicillin resistance may colonize the human oral cavity. Coagulase negative staphylococci were the majority in this limited study. Further studies are warranted to

[1]*Faculty of Science, University of Peradeniya, Peradeniya, Sri Lanka*
[2]*Ministry of Health, Colombo, Sri Lanka,*
[3]*Faculty of Dental Sciences, University of Peradeniya, Peradeniya, Sri Lanka.*

Address for correspondence: Professor J.A.M.S. Jayatilake, Faculty of Dental Sciences, University of Peradeniya, Peradeniya, Sri Lanka. Telephone: +94812397246 Email: sumedhaj@pdn.ac.lk
https://orcid.org/0000-0003-3961-4133

Received 23 October 2018 and revised version accepted 3 February 2019

determine the incidence of staphylococci in the oral cavity and their antimicrobial sensitivity.

***Key words**: mecA gene, Methicillin resistance, Oral cavity, Staphylococci*

Introduction

Staphylococci are important human commensals inhabiting the skin, nasal mucosa and the oral mucosa.[1-4] Staphylococci are notorious opportunistic pathogens that are responsible for the majority of hospital acquired infections worldwide.[5,6] While *S. aureus* is the leading pathogenic species, other species of coagulase negative staphylococci (CoNS) have also emerged as pathogens, especially in immunocompromised patients and patients with prosthetic devices.[7]

Several investigations support the fact that staphylococci are human oral colonizers both in health and disease. For instance, staphylococci have been abundantly isolated from the subgingival biofilm collected from patients with chronic periodontitis as well as from healthy individuals.[8-10] A recent analysis of subgingival biofilm collected from patients with chronic periodontitis as well as from healthy individuals identified both *S. aureus* and CoNS including *S. auricularis, S. epidermidis* and *S. saprophyticus* as oral microorganisms.[8] Staphylococci are also found to colonize removable partial dentures along with *Candida* and enteric bacilli.[11,12]

Development of antimicrobial resistance in staphylococci is a serious challenge faced by the clinicians.[5,6] In addition to the production of beta-lactamase, staphylococci generate antimicrobial resistance through the *mecA* gene that encodes penicillin binding protein-2a (PBP-2a) responsible for methicillin resistance.[13,14] PBP-2a has lower affinity for β-lactam antibiotics compared to the typical penicillin binding protein-2 (PBP2) produced by methicillin susceptible *S. aureus* (MSSA) as it blocks the active site from binding β lactams.[15,16] Consequently, staphylococci which carry chromosomally confined *mecA* gene are considered highly virulent due to their resistance to all β-lactam antibiotics.

Methicillin resistance in staphylococci is often detected by antimicrobial disc diffusion or broth dilution methods whereas detection of the *mecA* gene by PCR is a rapid and far more reliable technique.[17-20] Although methicillin resistance in staphylococci isolated from different human sources has been studied extensively[5], there are very few studies on methicillin resistance in oral staphylococci.[9] The purpose of this study therefore was to investigate methicillin resistance in oral staphylococci, using the CDDT and PCR for the detection of the *mecA* gene.

Materials and methods

Isolates of staphylococci

A total of 25 *Staphylococcus* isolates collected from the oral cavities of patients attending the Dental (Teaching) Hospital, Peradeniya, Sri Lanka were used for the study. These isolates were collected in an earlier study during which patients' informed consent was obtained to use such organisms for future research. The isolates

included 9 samples collected by subgingival plaque sampling and 16 samples collected using the concentrated oral rinse technique.[8] None of the samples were identifiable by the personal details of the patient. Ethical approval was obtained from the ethics review committee of the Faculty of Dental Sciences, University of Peradeniya.

Freeze-stored bacteria samples were recovered by culture on blood agar at 37 °C for 24-48 h. Identity of the bacteria was reconfirmed by cultural characteristics on blood agar, Gram stain, catalase and coagulase tests.

Cefoxitin disc diffusion test (CDDT)

The antibiotic sensitivity of staphylococci was tested using the CDDT following the Clinical and Laboratory Standards Institute (CLSI).[21] Standard suspensions of bacteria (0.5 McFarland) were prepared and inoculated onto Muller Hinton Agar (MHA) plates. After placing cefoxitin 30µg discs in the center of the plates, they were incubated at 37 ºC for 18-24h and the zones of inhibition were measured. For coagulase positive staphylococci (*S. aureus*), an inhibition zone diameter of ≤ 21mm was considered as methicillin resistant and ≥ 22mm was considered as methicillin sensitive whereas for CoNS, inhibition zone diameter of ≤ 24mm was considered as methicillin resistant and ≥ 25mm was considered as methicillin sensitive (CLSI M100[21]).

Extraction of DNA

The species characterization and demonstration of *mecA* gene in the genomic DNA of staphylococci were performed according to a method described previously with minor modifications.[22]

All 25 staphylococcal isolates, standard isolates of MSSA (ATCC 25923) and MRSA (ATCC 43300) were subjected to DNA extraction. Bacterial DNA was extracted from fresh bacterial cultures grown overnight on blood agar medium. From the fresh bacterial cultures, 3 to 4 loopfuls were harvested into 10mM TE buffer (10mM Tris-HCl pH, 7.5 /25mM EDTA) and subsequently washed twice with 10mM TE buffer. The resultant pellet after centrifugation was suspended in 0.6ml of 10mM TE buffer followed by addition of 10-20µl of lysozyme (50mg/ml) to the cell suspension and incubated at room temperature for 30min. The suspension was mixed gently after addition of 20µl of proteinase K (10mg/ml) and 60µl of SDS (10%) and the final suspension was incubated at 50 °C for 1h. The suspension was then mixed well with 0.6ml of phenol/chloroform and centrifuged at 13000rpm for 15min. 30µl of 5M NaCl was added to the aqueous layer extracted from the centrifuged product. This phenol/chloroform step was repeated once more with 10 min centrifugation and the resulting aqueous solution mixed with two volumes of absolute ethanol and centrifuged at 10000rpm for 5min. The supernatant was discarded, and the pellet washed with 70% ethanol. Finally, the DNA pellet was dried and dissolved with 50-100µl of TE buffer and stored at -20 ºC. The quality of the DNA was assessed by electrophoresis in 1% agarose gel.

Species characterization and the detection of *mecA* gene by multiplex PCR

16S rRNA gene amplification was performed as an internal control using the primers given in Weisburg *et al*.[23] Accordingly, FD1 (5'- AGAGTTTGATCCTGGCTCAG -3') and RD1 (5'- AAGGAGGTGATCCAGCC -3') primers were used to amplify the region of *16S rRNA* gene with the amplicon size of 1500bp.

For the amplification of *mecA* gene with the amplicon size of 532bp PCR was performed using the primers described previously.[17,22] The *mecA* locus was amplified using forward and reverse primers, (5'-AAAATCGATGGTAAAGGTTGG-3'/5'-AGTTCTGCAGTACCGGATTTGC-3') respectively. The amplifications were performed in 15µl reaction volumes each with 5µl of Taq mix (2X GoTaq Green® master mix reaction buffer [pH,8.5] with 400µM dATP, 400µM dATP 400µM dGTP, 400µM dTTP, 400µM dCTP, and 3mM $MgCl_2$), 0.5µl of each primer, 6ng templates DNA and nuclease free water. The reactions were carried out in a thermal cycler using the following program. Initial denaturation at 94 ºC for 5min, followed by 35 cycles of 1min of denaturation at 94 ºC, 1min of annealing temperature 55 ºC, 30 seconds of extension at 72 ºC and final extension at 72 ºC for 10min. The amplified PCR products were subsequently visualized on 1.5 % agarose gel stained with ethidium bromide (1µg/ml) for confirmation of PCR amplification. Finally, PCR products were visualized under UV and photographed.

Results

Identification of staphylococci

All 25 staphylococcus isolates were Gram positive, catalase positive cocci arranged in clusters. There were 7 (28%) coagulase positive staphylococcal (presumed *S. aureus*) isolates and the remaining 18 (72%) were coagulase negative staphylococci (CoNS). *16S rRNA* gene amplification results confirmed that all the isolates were staphylococci (Figure1). Both isolates which were identified as methicillin resistant by CDDT demonstrated the *mecA* gene by PCR whereas the remaining 23 isolates were negative for the *mecA* gene (Figure1).

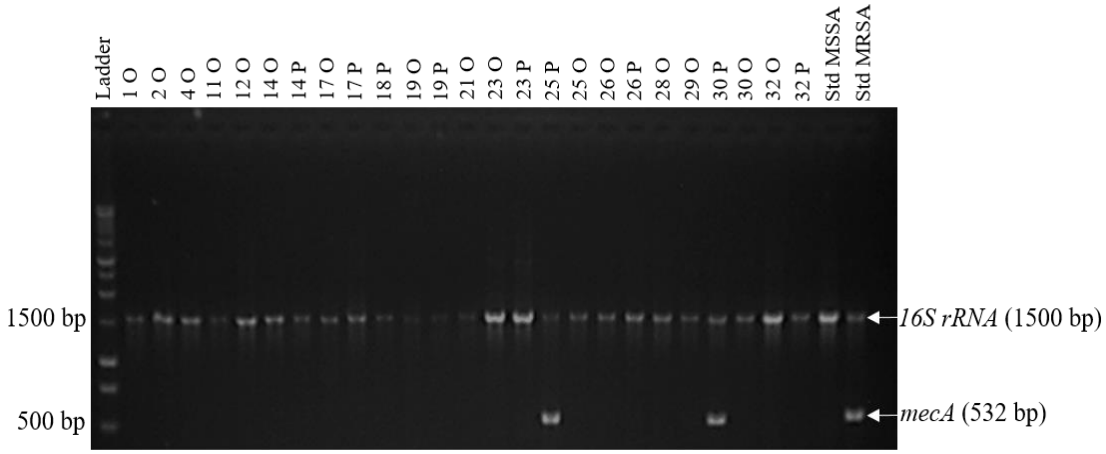


Figure 1 Gel image of the multiplex PCR using primer pairs for the amplification of *mecA* locus and the internal control *16S rRNA* locus, performed on all staphylococcal isolates, standard susceptible and resistant isolates.

Discussion

Although staphylococci are known to be frequent colonizers of the oral cavity, the incidence of methicillin resistance in oral staphylococci is poorly studied.[1,2,24] Hence, the current study investigated methicillin resistance and the responsible *mecA* gene in oral staphylococci isolated from a group of Sri Lankan patients.

Although a very limited study, the present study showed that the majority (72%) of oral staphylococci were CoNS supporting the previous findings of Loberto *et al*.[8] Only two of the coagulase negative staphylococci and none of the coagulase positive staphylococci in the present study were methicillin resistant. The present study shows that methicillin resistant staphylococci are found in the oral cavity of patients presenting to the Dental (Teaching) Hospital, Peradeniya, Sri Lanka. A retrospective analysis of data relevant to diagnostic oral microbiology in the UK showed that a small proportion (5%) of *S. aureus* isolated from oral specimens were MRSA.[2] However, these investigators did not report on the methicillin resistance of CoNS isolates. In contrast, another study that compared oral colonization of opportunistic pathogens including staphylococci in elderly Japanese patients with oral cancer and a healthy group showed that a large proportion, 9 of 13 oral *S. aureus* (69.2%) were MRSA. These investigators also demonstrated that 1 of 9 oral CoNS isolates (11.1%) were methicillin resistant.[25] Data obtained in the current study should be carefully interpreted due to the limited number of samples used in the analysis. Further studies using a larger sample would be beneficial to confirm the incidence of oral staphylococci and their antimicrobial resistance.

Both methicillin resistant isolates in the current study were collected from subgingival plaque samples of patients with chronic periodontitis lesions. Although some investigators[8-10] have isolated staphylococci from the subgingival biofilm collected from patients with chronic periodontitis as well as from healthy individuals, antimicrobial resistance of those staphylococci has not been adequately studied. Therefore, the detection of methicillin resistant isolates in subgingival plaque samples of patients with chronic periodontitis lesions warrants further investigations of antimicrobial resistance in staphylococci associated with periodontitis lesions.

As a phenotypic method for the detection of MR in staphylococci, the disc diffusion test was carried out using cefoxitin which is considered as the most reliable antibiotic for this purpose at present. Multiplex PCR assay was used to demonstrate the *mecA* gene in staphylococci. It has already been suggested that the detection of *mecA* gene with PCR offers rapid, simple, and accurate identification of methicillin resistance in staphylococci.[19] PCR and CDDT corroboration in this very limited study agrees with the previous reports that CDDT is in concordance with the PCR for demonstration of *mecA* gene.[20]

In conclusion, *S. aureus* and CoNS with or without methicillin resistance may colonize the human oral cavity as discussed above. Therefore, further studies with an increased sample size are warranted to confirm the exact prevalence of methicillin resistance in oral staphylococci.

Conflicts of interest: There are no conflicts of interest

References

1. Smith AJ, Jackson MS, Bagg J. The ecology of *Staphylococcus* species in the oral cavity. *J. Med. Microbiol*., 2001; 50:940-946. *doi: http://dx.doi.org/10.1099/0022-1317-50-11-940*
2. Smith AJ, Robertson D, Tang MK, Jackson MS, et al. *Staphylococcus aureus* in the oral cavity: a three-year retrospective analysis of clinical laboratory data. *Br. Dent. J.*, 2003; 195:701–703. *doi: http://dx.doi.org/10.1038/sj.bdj.4810832*
3. Kuehnert MJ, Kruszon-Moran D, Hill HA, et al. Prevalence of *Staphylococcus aureus* nasal colonization in the United States, 2001–2002. *J. Infect. Dis.*, 2006; 193:172–179. *doi: http://dx.doi.org/10.1086/499632*
4. Otto M. *Staphylococcus* colonization of the skin and antimicrobial peptides. *Expert Rev Dermatol*. 2010; 5:183–195. *doi: http://dx.doi.org/10.1586/edm.10.6*
5. Klein E, Smith DL, Laxminarayan R. Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*, United States, 1999–2005. *Emerg Infect Dis.* 2007; 13:1840-1846. *doi: http://dx.doi.org/10.3201/eid1312.070629*
6. Solberg CO. Spread of *Staphylococcus aureus* in hospitals: causes and prevention. *Scand J Infect Dis.* 2000; 32:587-95. *doi: http://dx.doi.org/10.1080/003655400459478*
7. Becker K, Heilmann C, Peters G. Coagulase-negative staphylococci. *Clin Microbiol Rev*. 2014; 27: 870–926. *doi: http://dx.doi.org/10.1128/CMR.00109-13*
8. Loberto JCS, de Paiva Martins CA, dos Santos SSF, et al. *Staphylococcus* spp. in the oral cavity and periodontal pockets of chronic periodontitis patients. *Braz. J. Microbiol*., 2004; 35:64-68. *doi: http://dx.doi.org/10.1590/S1517-83822004000100010*
9. Santos BRM, Demeda CS, Silva EENF, et al. Prevalence of subgingival *Staphylococcus* at periodontally healthy and diseased sites. *Braz. Dent. J*., 2014; 25: 271-276. *doi: http://dx.doi.org/10.1590/0103-6440201302285*
10. Souto R, de Andrade AFB, Uzeda M. Colombo APV. Prevalence of non-oral pathogenic bacteria in subgingival biofilm of subjects with chronic periodontitis. *Braz. J. Microbiol*., 2006; 37: 208-215. *doi: http://dx.doi.org/10.1590/S1517-83822006000300002*
11. Daniluk T, Fiedoruk K, Ściepuk M, et al. Aerobic bacteria in the oral cavity of patients with removable dentures. *Adv. Med. Sci*., 2006; 51: 86-90. *No doi*
12. Monroy TB, Moreno-Maldonado V, Franco-Martínez F, et al. *Candida albicans*, *Staphylococcus aureus* and *Streptococcus mutans* colonization in patients wearing dental prosthesis. *Med. Oral Patol. Oral Cir. Bucal.*, 2005; 10:27-39. *PMID:15800465*
13. Chambers HF. Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. *Clin. Microbiol. Rev*., 1997; 10:781-791. *doi: http://dx.doi.org/10.1128/CMR.10.4.781*
14. Lyon BR, Skurray R. Antimicrobial resistance of *Staphylococcus aureus*: *Genetic Basis Microbiol Rev*., 1987; 5:88-134. *No doi*
15. Lim D, Strynadka NC. Structural basis for the beta lactam resistance of PBP2a from methicillin-resistant *Staphylococcus aureus*. *Nat. Struct. Mol. Biol*., 2002; 9:870-876. *doi: https://doi.org/10.1038/nsb858*
16. Song MD, Wachi M, Doi M, et al. Evolution of an inducible penicillin-target protein in methicillin-resistant *Staphylococcus aureus* by gene fusion. *FEBS Lett*., 1987; 221: 167-171. *doi: https://doi.org/10.1016/0014-5793(87)80373-3*

17. Al-Ruaily MA, Khalil OM. Detection of (*mecA*) gene in methicillin resistant *Staphylococcus aureus* (MRSA) at Prince A / Rhman Sidery Hospital, Al-Jouf, Saudi Arabia. *J. Med. Genet. Genomics*. 2011; 3:41-45. *No doi*
18. Kipp F, Becker K, Peters G, von Eiff C. Evaluation of different methods to detect methicillin resistance in small-colony variants of *Staphylococcus aureus*. *J. Clin. Microbiol*., 2004; 42:1277-1279. *doi: http://dx.doi.org/10.1128/JCM.42.3.1277-1279.2004*
19. Strommenger B, Kettlitz C, Werner G, Witte W. Multiplex PCR assay simultaneous detection of nine clinically relevant antibiotic resistance genes in *Staphylococcus aureus*. *J. Clin. Microbiol*., 2003; 41:4089-4094. *doi: http://dx.doi.org/10.1128/JCM.41.9.4089-4094.2003*
20. Anand KB, Agrawal P, Kapila K. Comparison of cefoxitin disc diffusion test, oxacillin screen agar, and PCR for *mecA* gene for detection of MRSA. *Indian J. Med. Microbiol*., 2009; 27:27-29. *PMID: 19172055*
21. CLSI. *Performance Standards for Antimicrobial Susceptibility Testing.* 27th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2017. *No doi*
22. Martineau F, Francois J, Lansac N, et al. Correlation between the resistance genotype determined by multiplex PCR assays and antibiotic susceptibility pattern of *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Antimicrob. Agents Chemother*., 2000; 44:231-238. *doi: https://doi.org/10.1128/AAC.44.2.231-238.2000*
23. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol*., 1991; 173:697–703. *doi: https://doi.org/10.1128/jb.173.2.697-703.1991*
24. Costa DM, Kipnis A, Oliveira LSN, et al. Methicillin-resistant *Staphylococcus* sp. colonizing health care workers of a cancer hospital. *Braz. J. Microbiol*., 2014; 45: 799-805. *doi: http://dx.doi.org/10.1590/S1517-83822014000300006*
25. Yamashita K, Ohara M, Kojima T, et al. Prevalence of drug-resistant opportunistic microorganisms in oral cavity after treatment for oral cancer. *J. Oral Sci*., 2013; 55: 145-155. *doi: https://doi.org/10.2334/josnusd.55.145*

Research article

Establishing *Campylobacter* culture methods in a clinical diagnostic laboratory and the first report of *Campylobacter* species isolation in northern Sri Lanka

Mubarak FN[1], Pathirage MVSC[2], Kandasamy N[1], Keerthika A[1], Mahalingam S[1], Mithulan FJM[1], Selvi K[1], Umakanthan S[1], Perera KCR[3], Waidyarathne KDK[2], Sandeepani SAN[2], Sathiadas G[4], Arulmoli SK[5], Srisaravanapavananthan N[5]

Sri Lankan Journal of Infectious Diseases 2019 Vol.9 (1):49-58

DOI: http://dx.doi.org/10.4038/sljid.v9i1.8240

Abstract

Introduction: The Enteric Reference Laboratory of the Medical Research Institute (ERL/MRI), Colombo is the only public sector laboratory in Sri Lanka that performs *Campylobacter* cultures. Due to logistic limitations involving specimen transport from distant sites, efforts were taken to establish *Campylobacter* culture facilities in our local clinical microbiology laboratory.

Methods: A blood-free charcoal-based selective agar medium (Karmali medium) was chosen based on performance characteristics and quality control (QC)/verification performed at the ERL/MRI. A suitable incubating method was assessed and chosen, and QC was performed in our laboratory. A technical staff member of our local laboratory received capacity building training at the ERL/MRI.

Results: The quality control/verification process of the Karmali medium was satisfactory. The variable atmospheric incubator was chosen as the incubating method as it was shown to be more economical in the long-term given the anticipated work load and the QC was satisfactory. Following a satisfactory verification process, *Campylobacter* culture method was introduced in our laboratory. Five *C. jejuni* and one hippurate-negative *C. jejuni/C. coli* was detected in faecal specimens of six paediatric patients between May-December 2018. The isolation rate was 2.25% (6/267). Ciprofloxacin resistance was detected in four out of five *C. jejuni* isolates.

Conclusion: Establishing *Campylobacter* culture methods in a routine clinical diagnostic laboratory will be beneficial in regions with high prevalence of diarrhoeal disease and with logistic limitations for specimen transport to the central reference laboratory. This is the first report of

[1]*Microbiology Department, Teaching Hospital, Jaffna, Sri Lanka*
[2]*Enteric Reference Laboratory, Medical Research Institute, Colombo, Sri Lanka*
[3]*Medical Research Institute, Colombo, Sri Lanka*
[4]*Department of Paediatrics, Faculty of Medicine, University of Jaffna, Sri Lanka*
[5]*Paediatric Unit, Teaching Hospital, Jaffna, Sri Lanka*

Address for correspondence: Dr Nasmya Mubarak, 68A, Davidson Road, Colombo 4, Sri Lanka .

Telephone: +94 777518338 Email nsmya@yahoo.com https://orcid.org/0000-0002-0103-1189

Received 14 February 2019 and accepted 6 April 2019

isolation and antimicrobial susceptibility of *Campylobacter* species from patients in northern Sri Lanka.

Keywords: Campylobacter spp., Diarrhoeal disease, Stool culture, Blood-free charcoal-based selective agar, Variable atmospheric incubator

Introduction

Campylobacter species was identified as a significant enteric pathogen following the development of selective culture media by Butzler et al and Skirrow.[1] It is now recognized as a major cause of gastroenteritis worldwide, especially in younger children. A wide range of other gastrointestinal and extragastrointestinal manifestations have also been reported. The main causative species identified in human disease is *Campylobacter jejuni* followed by *Campylobacter coli.* Over the past decade the incidence and prevalence has increased worldwide. It is endemic in developing countries with reported isolation rates between 4-20%.[2]

The Enteric Reference Laboratory of the Medical Research Institute (ERL/MRI), Colombo is the only public sector laboratory in Sri Lanka that performs *Campylobacter* cultures. However there are many logistic limitations involving specimen transport from distant sites to the central reference laboratory. We detail the efforts taken to establish *Campylobacter* culture facilities in a clinical microbiology laboratory in a tertiary care hospital in Sri Lanka.

Methods

Identification

C. jejuni and *C. coli* are thermophilic, highly motile, Gram negative, curved, spiral or S-shaped bacilli requiring microaerobic atmospheric (5% O_2, 10% CO_2 and 85% N_2) conditions and 42 °C incubation temperature for optimum growth. Recommended duration of incubation is 48-72 hours.[3] Some authors report that 48-hour incubation is adequate for plates incubated in variable atmospheric incubators and 72-hour incubation may increase isolation rates if plates are incubated in jars.[4] Depending on the growth media used, grey, flat, irregular or spreading colonies are produced. Preliminary identification is by Gram stain, motility, oxidase test and catalase test. Oxidase positive colonies isolated on selective media incubated at 42 °C under microaerophilic conditions showing characteristic Gram stain morphology can be reliably identified as *Campylobacter* spp. Further identification tests include hippurate hydrolysis, indoxyl acetate hydrolysis, H_2S production, urease production, H_2 requirement, aryl sulfatase production, selenite reduction, growth in 1% glycine, growth at 25 °C and aero-tolerance. Hippurate hydrolysis can differentiate the most common species *C jejuni* (positive) from other *Campylobacter* spp. (negative) and no other tests are necessary for routine clinical purposes. *C. jejuni* and *C. coli* are biochemically identical with the exception of hippurate hydrolysis activity. Differentiation between rare hippurate hydrolysis negative *C. jejuni* strains and *C. coli* requires molecular methods; if unavailable, such isolates should be reported as hippurate-negative *C jejuni/C coli*.[3] (Table.1)

Table.1 Phenotypic properties of commonly encountered *Campylobacter* species of known or presumed clinical importance.

Organism	Catalase	H_2 required	Urease	H_2S (TSI)	Hippurate hydrolysis	Indoxyl acetate	Aryl sulfatase	Selenite reduction	Growth in 1%	Growth at 25 °C	Aerobic growth
C. jejuni subsp. *jejuni*	+	-	-	-	+	+	V	V	+	-	-
C. jejuni subsp. *doylei*	V	-	-	-	V	+	-	-	+	-	-
C. coli	+	-	-	V	-	+	-	+	+	-	-
C. fetus subsp. *fetus*	+	-	-	-	-	-	-	V	+	+	-
C. lari subsp. *lari*/*C. lari* subsp. *concheus*	+	-	V	-	-	-	-	V	+	-	-
C. upsaliensis	-	-	-	-	-	+	-	+	+	-	-

Adapted from "Useful phenotypic properties of *Campylobacter* and *Arcobacter* species"[3]

Selection of culture medium

Blood-based and charcoal-based selective agar media are used for *Campylobacter* isolation.[3] Blood-free, charcoal-based selective agars such as CSM (charcoal selective medium) and CCDA (cefoperazone charcoal deoxycholate agar) have better isolation rates and selectivity compared to the more commonly used blood-based selective agars such as Skirrow's medium and Butzler's medium. Of the available charcoal-based selective agars, Karmali's medium had the additional advantage of better differentiation.[5] Therefore Karmali medium (Oxoid, England) was chosen as the culture medium to be used in our laboratory.

Selection of incubation method

Microaerobic incubation methods for *Campylobacter* isolation include commercial gas generator kits used in jars, gas-jar evacuations followed by atmosphere replacement with bottled gasses and variable atmosphere incubators.[3] The cost of using gas generating sachets in a sealed jar was compared with the cost of using a variable atmosphere incubator based on estimates provided by local agents. The incubator method was selected over the gas-jar evacuation method based on the availability of local agents and long-term maintenance factors. Apart from the capital cost of the incubator, the projected cost for estimated daily usage of CO_2 and N_2 gas cylinders per month was calculated. Based on these findings the CO_2 incubator with the option to adjust O_2 control ranges to create hypoxic or hyperoxic culture conditions (HERAcell 150i,Thermo Scientific,Germany) was selected for use in our laboratory.

Technical training

A technical staff member of the local laboratory received capacity building training in enteric bacteriological diagnostics at the ERL/MRI, with special focus on *Campylobacter* diagnosis .

Quality control/verification of Karmali selective medium prior to introduction

A verification (quality control/QC) process was performed by testing the Karmali selective medium in parallel with the selective medium (Butzler's) used in the ERL/MRI for *Campylobacter* isolation and a non-selective blood agar control medium containing blood agar base and 5% sheep blood (5% SBA). Karmali medium was prepared using Karmali campylobacter agar base (Oxoid, England) and modified Karmali selective supplement (Oxoid, England) according to the manufacturers' recommendations. Testing protocols for QC were modified based on a method described by Gun-Munro et al. and included dilutions of control cultures, simulated faeces specimens and clinical specimens.[5]

The first phase consisted of testing a control strain of *C. jejuni* ATCC 33291 and a confirmed clinical isolate of *C. coli* by direct culture and by simulated positive faecal specimens. *C. jejuni* ATCC 33291 and *C. coli* available at the MRI are maintained at minus 70 °C in Tryptic Soy Broth containing 20% glycerol. These strains were subcultured on 5% SBA and incubated micro aerobically at 42 °C for 48 hours in sealed jars containing gas generating sachets to create an atmosphere containing 5% O2, 10% CO2 and 85% N2 to obtain a pure growth. 0.5 McFarland suspensions of these cultures was made from both *C. jejuni* and *C. coli* in phosphate buffered saline (PBS) and serial 10 fold dilutions were prepared from each of these working solutions. Faecal samples that were previously confirmed as negative for pathogenic enteric bacteria were emulsified in PBS and 0.5ml of neat and 10 fold diluted solutions of both *C. jejuni* and *C. coli* were inoculated to obtain homogenous suspensions. 20µl each of the neat and the 10-fold diluted solutions of the control cultures and the simulated faeces suspensions were then inoculated in parallel in Butzler's medium, Karmali medium and 5% SBA control in triplicate. All plates were incubated micro aerobically at 42 °C for 48 hours in sealed jars containing gas generating sachets to create an atmosphere containing 5%O2, 10%CO2 and 85%N2.

The second phase consisted of culturing routine clinical specimens received by the national enteric reference laboratory in parallel on both Butzler's medium and Karmali medium.

Quality control/verification of the variable atmospheric incubator

C. jejuni ATCC 33291 and *C. coli* isolates received by our laboratory from the ERL/MRI were subcultured on 5% SBA by incubating at 42 °C for 48 hours in sealed jars containing gas generating sachets to create an atmosphere containing 5% O2, 10% CO2 and 85% N2 . Each culture of *C. jejuni* and *C. coli* were inoculated as two sets in triplicate on Butzler's medium, Karmali medium and 5% SBA plate (control). One triplicate set of Butzler's, Karmali and 5% SBA plates were incubated in sealed jars containing gas generating sachets (CampyGen CN35, Oxoid, England) to create an atmosphere containing 5% O2, 10% CO2 and 85% N2. The other triplicate set was incubated in parallel in the variable atmospheric incubator available in our laboratory (Thermo Scientific, HERAcell 150i) with adjustment of the atmosphere to create 5% O2, 10% CO2 and 85% N2. All plates were incubated at 42 °C for 48 hours.

Introduction to the routine clinical bench

Following the satisfactory verification process, Karmali medium was introduced to the stool (faeces) culture bench in May 2018. Routine inoculation of faecal specimens from paediatric and

adult patients was done. Plates were incubated in the variable atmospheric incubator at 42 °C in microaerophilic conditions for 48 hours. *Campylobacter* isolates were identified by Gram stain, oxidase test, catalase test and motility testing. Hippurate hydrolysis was used to confirm *C. jejuni*. Antibiotic susceptibility testing was performed by disc diffusion method according to Clinical Laboratory Standards Institute (CLSI) guidelines (M-45, 3rd edition 2016)[6] which have been validated only for erythromycin, ciprofloxacin and tetracycline.

Results

The capital expenditure for the variable atmospheric incubator was 2,497,500.00 Sri Lankan rupees (including VAT). Table.2 is a comparison of the cost using gas generating sachets in sealed jars and the use of the variable atmosphere incubator using carbon dioxide and nitrogen cylinders. The estimated cost is calculated for a period of one month based on market prices at the time of writing.

Table 2: Cost comparison of using gas generating sachets in sealed jars and variable atmosphere incubator using carbon dioxide and nitrogen cylinders.

Item	Price	Unit price	Estimated usage per month	Cost per month*
CampyGen CN 35 (3.5L) Oxoid	6,730.00 (10 sachets/box)	673.00	30 sachets	20,190.00
Total cost for gas generating sachet/jar method				**20,190.00**
CO_2 gas cylinder (medical grade; 3Kg) (Ceylon Oxygen Ltd)	2,174,00	2174.00	Quarter cylinder	543.50
N_2 gas cylinder (4.5 grade; 47L 150 Bar) (Ceylon Oxygen Ltd)	2,890.50	2890.50	1 cylinder	2,890.50
Total cost for variable incubator method				**3,434.00**

*The estimated cost is calculated for a period of one month based on current market prices.

Quality control of Karmali selective medium

C. jejuni ATCC 33291 and *C. coli* isolation comparisons between Karmali selective medium, Butzler's selective medium and 5% SBA control are shown in Table 3. In the first phase of evaluation, growth of control cultures on Karmali selective medium was similar to the growth on the 5% SBA control. Isolation rates on Karmali selective medium were greater at higher organism dilutions when compared with Butzler's medium. In simulated faecal specimens, faecal flora suppression was greater, colonies showed typical appearance, and isolation rates of *Campylobacter* colonies were higher in Karmali medium compared to Butzler's medium. During the time period of this evaluation neither *C. jejuni* nor *C. coli* were isolated from any clinical specimens received by the enteric reference laboratory.

Table.3: *C. jejuni* ATCC 33291 and *C. coli* isolation comparison between Karmali selective medium, Butzler's selective medium and 5% SBA control

Suspension	Growth on 5% sheep blood agar control		Growth on Butzler's medium		Growth on Karmali medium	
	C jejuni ATCC 33291	*C coli*	*C jejuni* ATCC 33291	*C coli*	*C jejuni* ATCC 33291	*C coli*
Neat (0.5 McFarland)	+	+	+	+	+	+
10^{-1} dilution	+	+	+	+	+	+
10^{-2} dilution	+	+	+	+	+	+
10^{-3} dilution	+	+	+	+	+	+
10^{-4} dilution	+	+	+	+	+	+
10^{-5} dilution	+	+	-	-	+	+

Quality control of the variable atmospheric incubator

C. jejuni and *C. coli* isolation was comparable in the selective medium (Karmali and Butzler's) and growth on 5% SBA control medium was satisfactory on all plates that were incubated in parallel in the sealed jars containing gas generating sachets (CampyGen CN35, Oxoid, England) and the variable atmospheric incubator available in our laboratory (HERAcell 150i,Thermo Scientific, Germany).

Results following introduction to the routine clinical bench

Five *C. jejuni* and one hippurate-negative *C. jejuni/C. coli* were detected in faecal specimens from six paediatric patients between May-December 2018. The isolation rate was 2.25% (6/267). All isolates were sent for re-confirmation to the ERL/MRI.

Colonies on Karmali medium were round, grey, moist and semi-translucent. Faecal flora was significantly suppressed, and *Campylobacter* colonies were easily identifiable. All six isolates demonstrated typical 'S' shaped/spiral Gram stain appearance and motility. Five isolates were oxidase-positive and catalase-positive. These five isolates were confirmed as *C. jejuni* by hippurate hydrolysis and all were sensitive to erythromycin and tetracycline. Four *C. jejuni* were resistant and one was sensitive to ciprofloxacin. ERL/MRI confirmed the sixth isolate as oxidase-positive, catalase-negative and indoxyl-acetate-positive. This was reported as hippurate-negative *C. jejuni/C. coli* which was sensitive to all three antibiotics tested. (Figures 1and 2)

All patients were ≤ 2 years of age. One patient had a mixed infection with *Salmonella* Chester. *Salmonella* Chester was isolated in both faeces and blood of this patient.

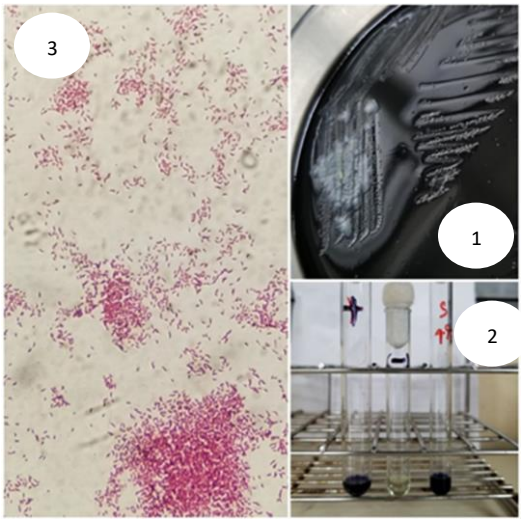


Figure 1
1.*Campylobacter* species colonies isolated from a faecal specimen on Karmali selective medium
2.Hippurate hydrolysis test
3.Gram stain

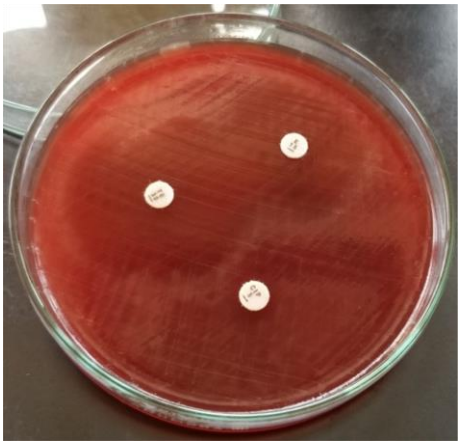


Figure2
Campylobacter ABST on Mueller-Hinton agar with 5% sheep blood

Discussion

The first report of *Campylobacter* isolation in Sri Lanka was by Palasuntheram et al in 1982 and all three isolates were identified as *C. jejuni*.[7] The same authors reported a total of nine isolates of *C. jejuni* by the year 1984.[8] Studies by Pathirage[9], Cooray et al[10], and Udugama[11] and Vidyarathne et al[12] reported isolation rates of *Campylobacter* species as 9.36%, 2.5-6.7%, 3.8% and 8.82% respectively. *C. jejuni* and *C. coli* were the only species identified and the predominant species was *C. jejuni*. The overall ciprofloxacin resistance rate reported by Pathirage[9], Cooray et al[10], Udugama[11] and Vidyarathne et al[12] was 43.9%, 35.2%, 100% and 83.3% respectively.

Our laboratory received 2092 faecal specimens between June-December 2018. Significant *Shigella* species isolation has been noted over the years.[13] However a large number of faecal specimens were culture-negative for other common enteric pathogens such as *Salmonella* spp, *Vibrio* spp. and pathogenic *E.coli*.[13] Detection of *Aeromonas*, *Plesiomonas* and *Yersinia* cannot be commented upon as they are not included in the current routine diagnostic workup. It was considered important to introduce routine *Campylobacter* culture methods in our clinical laboratory due to the high prevalence of diarrhoeal diseases in the region and the logistic limitations of being situated more 400km away from the central reference laboratory.

Early studies recommend the use of more than one selective culture medium to optimize isolation rates of *C. jejuni* and *C. coli*.[1,14,15] Based on studies done by Karmali et al[1] and Gun-Munro et al[5], a charcoal-based selective medium is recommended for use as a single medium, especially in

resource-limited developing countries. Satisfactory isolation rates, along with greater suppression of normal faecal flora and enhanced recognition of *Campylobacter* colonies were observed in charcoal-based media and the medium described by Karmali et al showed optimum performance. As shown by our cost estimations, the use of a variable atmospheric incubator was more economical in the long-term despite the initial capital cost. This is especially useful for regional laboratories that process a large number of specimens. Our clinical microbiology laboratory is based in the only tertiary care hospital of the Northern Province and also serves as a regional laboratory for four main peripheral hospitals within the Province. Consideration of the projected workload and logistic problems related to distance from ERL/MRI, led to the investment in a variable atmospheric incubator. We chose Karmali charcoal-based selective medium as the single culture medium based on published performance criteria[1,5] and the ease of preparation of a non-blood based medium in a routine clinical laboratory.

Despite *Campylobacter* species not being isolated from any clinical specimens (in both Butzler and Karmali media) during the second phase of evaluation at ERL/MRI, the satisfactory performance of Karmali medium during the first phase was considered adequate for an introductory trial in our routine diagnostic service. The isolation of six *Campylobacter* isolates (five *C. jejuni* and one hippurate-negative *C. jejuni/C. coli*) during a six month period indicates the success of the methods introduced and prevalence of the disease. Although all isolates were sensitive to erythromycin and tetracycline, ciprofloxacin resistance was detected in 4 of the 5 *C. jejuni* isolates. Fluoroquinolone-resistant *Campylobacter* spp. has been identified as one of the high priority pathogens in the WHO priority pathogens list for R&D of new antibiotics.[16] This highlights the importance of isolation and determination of antimicrobial susceptibility of *Campylobacter* spp. for treatment and surveillance purposes. This is the first report of isolation and antimicrobial susceptibility of *Campylobacter* species from patients in northern Sri Lanka.

Future approach

Studies by Endtz et al and Besse`de E et al indicated that the true incidence of *Campylobacter* enteritis could be underestimated if antibiotic containing selective culture media are used as the only method of diagnosis as this will inhibit *Campylobacter* species with variable antibiotic susceptibilities.[2,3.17,18] Incubating conditions (temperatures and gas composition) will also affect the isolation rates of various thermophilic and non-thermophilic *Campylobacter* species and those species that require H_2 for primary isolation.[2,3] Currently both culture-dependent and/or culture-independent (serological and molecular) methods are being used worldwide for laboratory diagnosis of *Campylobacter* species.[2,3] There is no gold standard method that will enable the detection of all probable *Campylobacter* human pathogens due to limitations of each method.[2] Membrane filtration techniques, enzyme-immuno assays (EIA-stool antigen tests) and molecular diagnostic methods have detected a wide-range of *Campylobacter* species as causative agents in human disease.[17.18] A South African study in children with diarrhoea using the membrane filtration culture technique showed that only 40% of *Campylobacter* species isolated were *C. jejuni*.[19] The Centres for Disease Control and Prevention (CDC) defines a confirmed case as a culture positive case and a probable case as detection of *Campylobacter* species using a culture-independent diagnostic test (CIDT) such as PCR and recommends culture confirmation (knowns as reflex culture) for these cases.[20] Quantitative PCR alone or PCR and EIA have been suggested as

alternatives for culture.[21] The use of CIDT as stand-alone tests are still being evaluated as the sensitivity, specificity and positive predictive value of these assays are reported to be variable.[20] M'ikanatha et al showed a variation between laboratories in testing methods used for *Campylobacter* detection in human stool samples and has recommended that laboratory practice guidelines for *Campylobacter* testing should be developed to ensure uniformity between laboratories and optimal case detection rates.[22] However isolation of *Campylobacter* will remain important for antimicrobial susceptibility testing and precise identification.[20] In countries with endemic high antimicrobial resistance rates this is essential for surveillance and treatment.[18,20] Therefore while culture techniques will continue to remain a main diagnostic feature, the introduction of CIDT for better case detection rates will need to be considered in the future.

Conflicts of Interests: There are no conflicts of interest

Ethical statement: As this was a laboratory based capacity building activity for routine diagnostics, ethical clearance was not indicated.

Acknowledgements: We acknowledge the officials and staff of the Deputy Director General/Laboratory Services office and the Medical Supplies Division of the Ministry of Health, Nutrition & Indigenous Medicine, Sri Lanka for the support and funding of this initiative.

Funding: All equipment, reagents and consumables were procured through the routine funding allocation and supplies from the Ministry of Health, Sri Lanka.

References

1. Karmali MA, Simor AE, Roscoe M, et al. Evaluation of a blood-free, charcoal-based, selective medium for the isolation of *Campylobacter* organisms from feces. *J. Clin. Microbiol*. 1986; 23(3):456-459. *No doi PMID: 3958143*
2. Kaakoush NO, Castaño-Rodríguez N, Mitchell HM, et al. Global epidemiology of *Campylobacter* Infection. *Clin Microbiol Rev*. 2015; 28(3):687-720. *doi: 10.1128/CMR.00006-15.*
3. Fitzgerald C, Nachamkin I. *Campylobacter* and *Arcobacter*. In: Jorgensen JH, Pfaller MA, Carroll KC, et al. (eds). Manual of Clinical Microbiology (11th ed). Washington: ASM Press, 2015: pp 998–1012.
4. Goossens H, Butzler JP. Isolation of *Campylobacter spp*. from stool specimens with a semisolid medium. *J Clin Microbiol*. 1991; 29(11):2681-2682. *No doi. PMID: 1774290*
5. Gun-Munro J, Rennie RP, Thornley JH, et al. Laboratory and clinical evaluation of isolation media for *Campylobacter jejuni*. *J Clin Microbiol*. 1987; 25(12):2274-2277. *No doi. PMID: 3429621*
6. Clinical and Laboratory Standards Institute. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. M45 (3rd Ed). Wayne: CLSI, 2016.
7. Palasuntheram C, Wijesekera DS. Campylobacter Enteritis in Sri Lanka. *Ceylon Med J*. 1982; 27(2):80-84. *No doi. PMID: 7160007*
8. Palasuntheram C, Wijesekera DS. A study of *Campylobacter fetus* subsp. *jejuni* strains isolated from the stools of diarrhoeic children in Sri Lanka. *Trans R Soc Trop Med Hyg*. 1984; 78:173-174. *No doi PMID: 6464104*

9. Pathirage MVSC. Incidence and Other Related Factors of Campylobacter Species Among Patients with Diarrhoea Presenting to Lady Ridgeway Children's Hospital. MD (Medical Microbiology) Dissertation Thesis; Postgraduate Institute of Medicine, University of Colombo, Sri Lanka, 2004.
10. Cooray KJ, Perera KCR. Campylobacter surveillance in Sri Lanka. *The Bulletin of the Sri Lanka College of Microbiologists* (Abstract). 2007; 5(1):20-21.
11. Udugama SG. Detection of common viral and bacterial enteric pathogens from children with diarrhoea attending the Lady Ridgeway Children's Hospital. MD (Medical Microbiology) Dissertation Thesis; Postgraduate Institute of Medicine, University of Colombo, Sri Lanka, 2011.
12. Vidyarathne GGK, Pathirage S, Silva TJ, et al. Prevalence of Campylobacter species and their antibiotic sensitivity pattern among patients with diarrhoea in a paediatric tertiary care hospital in Sri Lanka. *The Bulletin of the Sri Lanka College of Microbiologists* (Abstract). 2018; 16(1):39-40.
13. Mubarak FN, Pathirage MVSC, Shayshananth T, et al. Speciation, serotyping and antimicrobial susceptibility pattern of *Shigella* isolates in northern Sri Lanka. *Am J Trop Med Hyg*. 2017; 97(5) Suppl:448 *doi: https://doi.org/10.4269/ajtmh.abstract2017*
14. Bolton FJ, Coates D, Hinchcliffe PM et al. Comparison of selective media for isolation of Campylobacter jejuni/coli. *J Clin Pathol*. 1983; 36(1):78-83. *No doi*. *PMID: 6822680*
15. Patton CM, Mitchell SW, Potter ME et al. Comparison of selective media for primary isolation of *Campylobacter fetus* subsp. *jejuni*. *J Clin Microbiol*. 1981; 13(2):326-330. *No doi. PMID: 7204549*
16. World Health Organization. WHO publishes list of bacteria for which new antibiotics are urgently needed. Geneva: World Health Organization, 2017. *https://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed*
17. Endtz HP, Ruijs GJ, Zwinderman AH, et al. Comparison of six media, including a semisolid agar, for the isolation of various Campylobacter species from stool specimens. *J. Clin. Microbiol*. 1991; 29(5):1007-1010. *No doi PMID: 2056033*
18. Besse`de E, Delcamp A, Sifre´ E, et al. New methods for detection of campylobacters in stool samples in comparison to culture. *J Clin Microbiol.* 2011; 49(3):941-944. *doi: 10.1128/JCM.01489-10.*
19. Platts-Mills JA, Kosek M. Update on the burden of Campylobacter in developing countries. *Curr Opin Infect Dis*. 2014; 27(5):444-450. *doi: 10.1097/QCO.0000000000000091*.
20. Centers for Disease Control and Prevention: Campylobacter (Campylobacteriosis). Atlanta: Centers for Disease Control and Prevention, 2017. *https://www.cdc.gov/campylobacter/index.html*
21. do Nascimento Veras H, da Silva Quetz J, Lima IFN, et al. Combination of different methods for detection of *Campylobacter spp*. in young children with moderate to severe diarrhea. *Journal Microbiol Methods*. 2016; 128:7–9. *doi: 10.1016/j.mimet.2016.06.026*
22. M'Ikanatha NM, Dettinger LA, Perry A, et al. Culturing stool specimens for *Campylobacter spp*., Pennsylvania, USA. *Emerg Infect Dis*. 2012; 18(3):484-7. *doi: 10.3201/eid1803.111266*.

Research article

Morphometrics of amastigote forms of *Leishmania donovani* in cutaneous leishmaniasis patients in Sri Lanka: evidence for the presence of promastigote-like structures

A Gunasekara[1], D Iddawela[1], S Wickramasinghe[1]

Sri Lankan Journal of Infectious Diseases 2019 Vol.9 (1):59-67

DOI: http://dx.doi.org/10.4038/sljid.v9i1.8238

Abstract

Objective: Investigations have not yet been carried out to identify the different morphological forms of amastigotes present in patients with cutaneous leishmaniasis (CL) in Sri Lanka. Thus, this paper describes the existence of different amastigote forms in cutaneous lesions for the first time in Sri Lanka.

Methods: This was a retrospective study. One hundred and thirty skin smears were investigated to identify the different morphometric forms of *L. donovani*. In addition, demographic data (age, gender, occupation, household characteristics, and geographic area) were analyzed using department records.

Results: Of the 130 samples, 84 (60.83%) samples had amastigote forms. Three (2.31%) samples had amastigotes in intracellular locations while 43 (33.08%) had amastigotes extracellularly. Nineteen (14.62%) samples had amastigotes in intracellular and extracellular locations simultaneously. Promastigote-like structures (PLS) were found in 65 (50%) samples. Of the 65 samples, 19 (14.62%) had both PLS and amastigote forms. PLS alone (no association with amastigote forms) were found in 46 (35.38%) samples. Amastigotes were found predominantly in lesions <2 months old while PLS were more common in 8 to 12 months old lesions.

Conclusion: Microscopic examinations of skin smears revealed the presence of promastigote-like structures for the first time in patients with CL in Sri Lanka. Therefore, we suggest that different morphometric features of amastigotes should not be ignored as they may be useful in diagnosis of CL in clinically suspected patients.

[1]*Department of Parasitology, Faculty of Medicine, University of Peradeniya, Sri Lanka*

Address for correspondence: Prof. S. Wickramasinghe, Department of Parasitology, Faculty of Medicine, University of Peradeniya Telephone: +94770074544 Email: susijijp@yahoo.co.jp

https://orcid.org/0000-0003-2046-1093

Received 21 January 2019 and revised version accepted 29 March 2019

Keywords: *Cutaneous leishmaniasis, Leishmania donovani, Amastigote, Morphometrics, Promastigote-like structures, Chronicity, Sri Lanka*

Introduction

Leishmaniasis is a vector-borne parasitic disease caused by an obligate intracellular protozoan of the genus *Leishmania.* This disease is endemic in 66 Old World and 22 New World countries in the tropics, subtropics and southern Europe.[1,2,3,4] According to World Health Organization (WHO) estimates, 1.5 million cutaneous leishmaniasis cases and 500,000 visceral leishmaniasis cases occur annually and 12 million people were infected globally.[5] Thus, leishmaniasis is considered as an emerging and re-emerging infectious disease due to its wider geographical distribution with a high incidence.[5] Leishmaniasis is capable of causing a spectrum of clinical syndromes ranging from cutaneous ulcerations to systemic infections - cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL) or "kala-azar". In addition, fourth (diffused cutaneous leishmaniasis (DCL) and fifth forms (post-kala-azar dermal syndrome (PKDS) have also been described.[6,7]

The infective stage (promastigote) is transmitted to mammals via the bite of an infected female sandfly of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World.[8] Congenital and blood-borne transmissions have been reported.[9,10,11,12] Humans are generally considered to be an incidental host for leishmaniasis.[13]

In Sri Lanka, the first autochthonous leishmaniasis case was reported in 1992.[14] Since then, over 2500 CL cases have been reported islandwide.[15] Leishmaniasis was declared a notifiable disease by the Ministry of Health, Sri Lanka in 2006. Cutaneous leishmaniasis is the main form of the disease reported in Sri Lanka.[16] In addition, several VL[17] and atypical MCL[18] cases have been reported. *Leishmania donovani* (*L. donovani*) zymodeme MON-37 is responsible for CL in Sri Lanka[19] and the likely vector is *Phlebotomus argentipes* sibling species A.[20]

Diagnosis of CL in Sri Lanka is mainly based on direct microscopy. The occurrence of different morphometric forms of *L. donovani* amastigotes in CL patients has not yet been investigated in Sri Lanka. Therefore, the present study was carried out to identify the different morphometric forms of *L. donovani*.

Methods

Data and sample collection

This was a retrospective study. One hundred and thirty skin smears stored in the Department of Parasitology, Faculty of Medicine, University of Peradeniya were investigated to identify the different morphometric forms of *L. donovani*. In addition, demographic data (age, gender, occupation, household characteristics, and geographic area) were analyzed using department records. The presence of different forms of the amastigote was recorded.

Results

Demographic analysis

Hundred and thirty clinically suspected CL patients were referred from 8 districts (Kegalle, Kurunegala, Anuradhapura, Polonnaruwa, Puttalam, Jaffna and Matale) for diagnosis. All suspected patients were smear-positive for CL. Of them, 73 (56.15%) were males and 57 (43.85%) were females. Categorized by age, 56 (43.08%) patients were between 16-35 years and 53 (40.77%) between 36-60 years with 12 (9.23%) less than 15 years. The highest incidence of CL was reported from patients who were involved in outdoor activities (47.69%) followed by the unemployed group (44%). Patients confined to indoor activities had fewer infections (18.6%). Patients over 60 years were the least affected group (6.92%). Of the study population, 9.23% were living close to scrub jungles and 18.46% were associated with thatched or mud sheds. Domestic animals were present in the premises of 28 (21.54%) patients (Table 1).

Table 1: Demographics of CL patients

Demographics	No. of patients	Percentage (%)
Sex		
Male	73	56.15
Female	57	43.85
Age		
<15	12	9.23
16-35	56	43.08
36-60	53	40.77
>60	9	6.92
Occupation		
Outdoor	62	47.69
Indoor	24	18.46
Unemployed	44	33.85
Risk Factors		
Scrub jungle nearby	12	9.23
Ownership of domestic animals (cattle, goats etc.)	28	21.54
Thatched or mud sheds near by	24	18.46

Table 2: Distribution of lesions

Site of infection	No. of patients	Percentage (%)
Head (face and ears)	37	28.46
Neck	6	4.62
Upper limbs	46	35.38
Trunk	4	3.08
Lower limbs	24	18.46
Foot	8	6.15
Multiple sites	5	3.85

Distribution of skin lesions

Of the 130 patients, 35.38% had lesions on the upper limbs that were the most commonly affected body part. The second commonest place was the head (face and ears) (28.46%). Lesions on lower limbs were found in 18.6% of patients. The least affected area of the body was the trunk (3.08%). Four patients had lesions on the trunk and 3 of them were males while one was a female. Five patients (3.85%) had lesions on multiple sites and among them one patient had severe ulcerative lesions on the neck extending towards the trunk (Table 2).

Morphometrics of amastigote forms

All samples obtained from suspected CL patients (n=130) had amastigotes and/or promastigote-like structures (PLS). Amastigote forms alone were present in 65 (50%) patients and they were found in both intracellular and extracellular locations (Figures 1 and 2). Pseudocysts (a number of amastigotes in one cyst) can be clearly seen in Figure 1. The majority of patients (62) had extracellular amastigotes. Three patients had intracellular amastigotes while 19 patients had both

intracellular and extracellular located amastigotes. Amastigotes found in this study were either round (25.00%), oval (32.14%) or spindle (42.85%) in shape. The sizes of the amastigotes ranged from 2 µm to 5 µm (Figure 2).

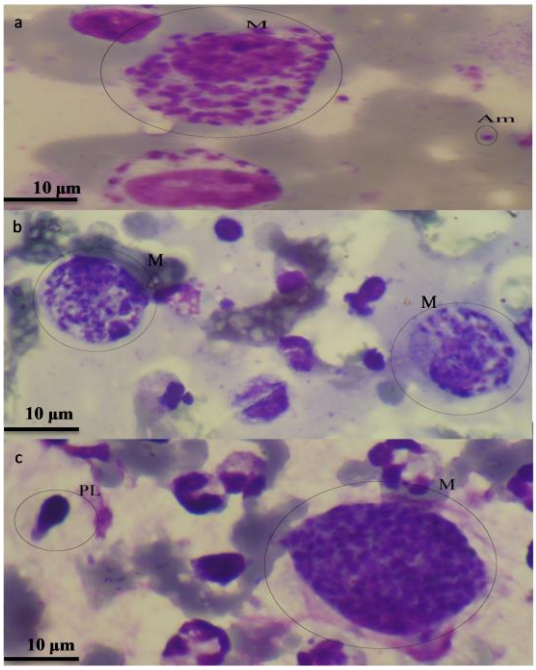


Figure 1: Skin smears stained with Giemsa (a, b and c).

Am - amastigote; M - pseudo cysts;
PL - promastigote-like structure.

Different morphometric features of the amastigotes that deviated from the typical shape (round or oval) are described for the first time in CL patient in Sri Lanka. This particular form was termed as "promastigote-like structures" (PLS). Promastigote-like structures were 6-35 µm in size (Figure 3). They all had a tail-like structure. Both PLS and amastigotes were seen in 19 (16.42%) patients. Promastigote-like structures alone were identified in 46 (35.38%) patients. Amastigotes alone were identified in patients who had lesions of less than two months duration. Patients with 2 to 8 month old lesions had both amastigotes and PLS. Over 9 months old lesions had only PLS (Table 3).

Table 3: Comparisons between duration of lesions and the presence of amastigotes and/or promastigotes-like (PLS) structures in CL patients

Duration (months)	Amastigotes alone (%)	Amastigotes+PLS (%)	PLS alone (%)
<2	43 (33.08)	-	-
3 to 5	19 (14.62)	7 (5.38)	-
6 to 8	3 (2.30)	12 (9.23)	-
9 to 12	-	-	38 (29.23)
>12	-	-	8 (6.15)

PLS; promastigote-like structure, n=130

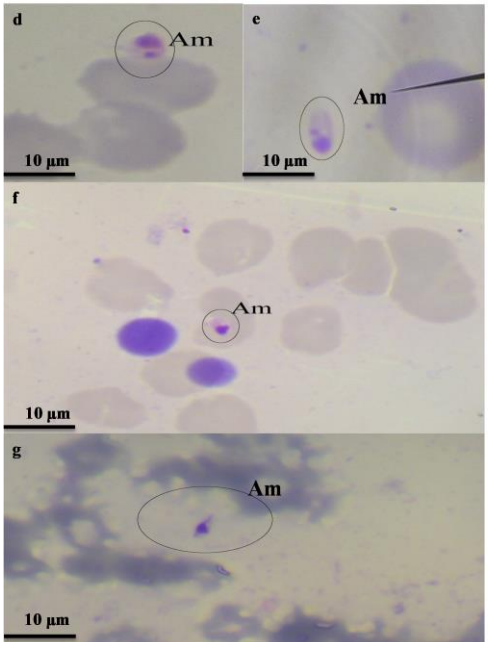


Figure 2: Different morphometrics of the amastigote.

Am – amastigote, d and f – round shaped, e – oval shaped, g – spindle shaped

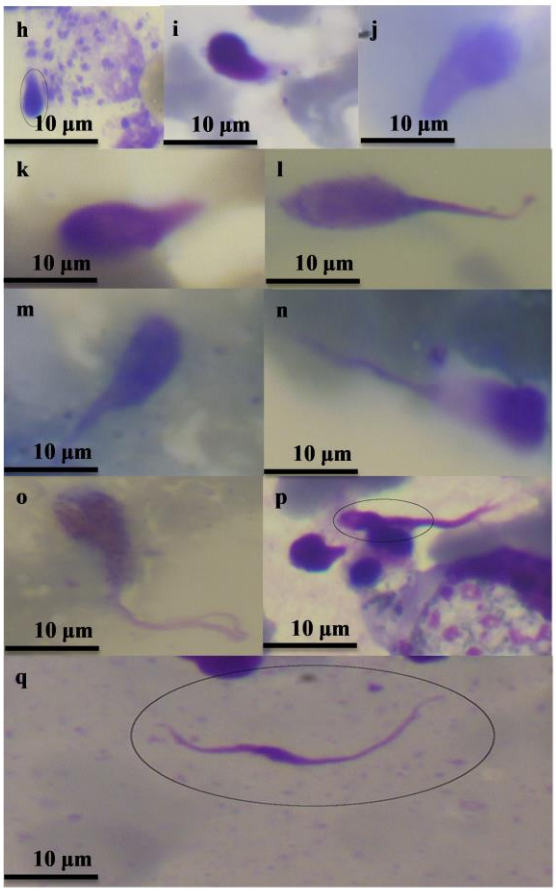


Figure 3: Transformation into promastigote-like structures.

h,i and j : condensed chromatin, cytoplasm disappears, grow larger and having a tail-like structure on one end.
k, l and m : candle flame shaped.
n, o and p : further growth of the tail-like structure.
q : transformation into fiber-forming stage (Daboul 2008)

Discussion

Demographic profile

Of the 130 CL cases, the majority were reported from Kandy (43.07%), Kegalle (17.69%), Matale (14.62%) and Kurunegala (13.08%) respectively. In this study, the spatial distribution of CL cases was expected to be biased since most of the patients referred to our laboratory were from Kandy, Kurunegala, Kegalle and Matale Hospitals. Nevertheless, previous studies and surveillance data have shown that the incidence of CL was much higher in the districts of Anuradhapura, Polonnaruwa, Hambanthota, Matara, Kurunegala, and Mullaitivu compared to that of other districts in Sri Lanka.[15,21,22,23,24]

According to the records, different cutaneous manifestations such as papules, nodules, and volcanic ulcers were present. Some CL patients had multiple lesions (29.23%). The number of lesions found in one patient ranged from 2 to 5 as reported by Nawaratna *et al.*, (2007) previously.[25]

It has been shown that uncovered parts of the body were more prone to sandfly bites than covered areas.[25,26,27] This may be the reason why lesions were often found in uncovered parts of the body. Male patients had a higher positivity rate (56.15%) than females (43.85%). This may be attributed to the increased outdoor activities, clothing preferences, occupation and other behavioral habits of these males. The duration of cutaneous lesions in CL patients were varied, 99.23% of whom had between weeks to 3 year old lesions. Only one patient had lesions of more than 6 years, which many have been due to treatment failures or reinfection.[25,28]

Cellular locations of amastigotes

Previous studies have shown amastigote forms of *Leishmania* species as an obligate intracellular protozoan.[29] However, contrary to previous investigations, research carried out by Daboul (2008) showed that *Leishmania* species do not only exist as an obligate intracellular parasite. Furthermore, the same study has shown the occurrence of amastigotes in both intracellular and extracellular locations.[30] In the present study as well, we have identified the amastigotes in both locations (Figures 1 and 2) in CL patients in Sri Lanka. Therefore, our study provides further evidence to prove that amastigotes exist in both intracellular and extracellular locations as shown by Daboul (2008).[30] It seems that amastigotes can survive in extracellular locations though the environment is not necessarily the same as in mononuclear cells. Based on those findings, the previous authors have speculated that *Leishmania* may no longer be considered as an obligate intracellular parasite, though more in depth investigations would be necessary to confirm such assumptions.[30]

Morphometrics of amastigote forms

We found different morphometric features of amastigotes (Figure 3) for the first time in CL patients in Sri Lanka. They were observed only in the extracellular locations. Daboul (2008) showed that the amastigote transforms into an ovum containing "promastigote embryo-like structure" once it comes out from the macrophage.[30] This ovum containing embryo-like structure is spindle-shaped, has a central nucleus surrounded by the cytoplasm (Figure 2, g). Daboul (2008) also suggested that the nucleus of amastigotes moves to one pole of the amastigote and the cytoplasm pushes the other pole in such a way leading to condensation of chromatin.[30] In the next stage of the development, the embryo grows larger in size and chromatin gets more condensed. Daboul (2008 and 2011) hypothesized this phenomenon as a "candle flame appearance" (Figure 3), while the cytoplasm is gradually reduced in size and disappeared.[30,31] The embryo continues to grow and form a tail-like structure. This tail-like structure develops further into flagellae and its appearance is quite similar to the promastigote (Figure 3). In addition, all other transformation stages that were described by Daboul (2008 and 2011) and Sharquie *et al.*,(2002) were found in our study as shown in Figures 1, 2 and 3.[30,32] Gradually, the flagella of promastigote-like structures get more condensed, thickened and enlarged up to 40 μm in length or more and Daboul (2008) described this process as transformation into a fibre–forming stage.[30] Sharquie *et al.*,(2002) have shown the presence of a fibre forming stage in the dry nodular type lesion.[32] This process may lead to an inflammatory fibre granulomatous immune reaction associated with lymphocytes, mononuclear cells, and a variable number of plasma cells.[30,32] At the end of this process, fibres are elongated and thickened from both sides, while the nucleus is condensed into the middle, getting thinner and smaller (Figure 3, p-q). At this point, a few plasma cells and lymphocytes can be seen, and the lesion is dry and about to heal leaving a permanent scar.[30] Daboul (2008) has suggested that the post healing lifetime scar may be due to pseudo-fibres of the parasite itself and not as a result of the human fibroblasts.[30]

In Sri Lanka, diagnosis of CL has been carried out routinely using clinical symptoms along with direct microscopy with the presence of amastigotes confirming the diagnosis and absence of same excluding CL. However, the sensitivity of the direct microscopic method is only 60-70%.[33] Therefore over 30% - 40% of cases may not be diagnosed by direct microscopy. The low sensitivity of the direct microscopic method may be due to the progression of the disease and low parasite counts in the lesion.[34] According to a recent study, amastigotes may also not be found in clinically hyperactive and inflamed CL lesions.[35] Also, it further suggested that amastigotes could be able to survive in the extracellular fluid. Environmental conditions in extracellular fluid are harsher than within macrophages. In order to survive in such conditions, amastigotes might have transformed into promastigote-like forms**.** As Daboul (2013) has described, promastigote-like structures become active and penetrate the subcutaneous tissues and cause the real signs and symptoms of the disease at this stage.[35] The morphometric features of promastigote–like structures and candle flame form are active forms of amastigotes that are transformed into fibres gradually. Similar morphometric features were identified in CL patients in Sri Lanka. Amastigotes were frequently found in patients who had less than 5 month old lesions. This finding suggests that amastigote forms are more common at the early stages of disease progression and promastigote-like structures can be found in later stages (Table 3).

Conclusions

Microscopic examination of skin smears showed the presence of promastigote-like structures for the first time in CL patients in Sri Lanka. Amastigotes were predominantly present at the early stages of cutaneous lesions while promastigote-like structures were more commonly seen in older lesions. Therefore, we suggest that different morphometric features of amastigotes should not be ignored as they may be useful in the diagnosis of CL in clinically suspected patients.

Limitations of the study

Identification of different forms of the amastigotes was done using the light microscope only. The use of a transmission electron microscope or other molecular techniques such as DNA analysis would have been useful to identify the different forms of these amastigotes.

Acknowledgments

We would like to thank Mr. Sunil Wijesundera and Mrs. Lakmali Bandara, Department of Parasitology, Faculty of Medicine, University of Peradeniya, Sri Lanka for providing technical assistance during the study.

References

1. Desjeux P. Leishmaniasis, public health aspects and control. *Clin Dermatol*1996; 14:417-23. *doi: https://doi.org/10.1016/0738-081X(96)00057-0*
2. Herwaldt BL. Leishmaniasis. *Lancet* 1999; 354:1191-99. *doi: 10.1016/S0140-6736(98)10178-2*
3. Koutis CH. Special Epidemiology. Editions, Technological Educational Institute of Athens. Athens, Greece 2007.

4. Dujardin JC, Campino L, Cañavate C, et al. Spread of vector-borne diseases and neglect of Leishmaniases in Europe. *J Emer Infec Dis* 2008; 14:013-8. *doi: 10.3201/eid1407.071589*
5. WHO. Leishmaniasis surveillance and control of leishmaniasis. Available from: *http://www.who.int/entity/leishmaniasis/surveillance/en/.2014.*
6. Bañuls A, Hide M, Prugnolle F. *Leishmania* and the Leishmaniases: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. *Adv Parasitol* 2007; 64:2-70. *doi: https://doi.org/10.1016/S0065-308X(06)64001-3*
7. Salotra P, Sreenivas G, Pogue GP, et al. Development of a species-specific PCR assay for detection of *Leishmania donovani* in clinical samples from patients with Kala-Azar and post-Kala-Azar dermal Leishmaniasis. *J Clin microbiol* 2001; 39:849-57. *doi: 10.1128/JCM.39.3.849–854.2001*
8. Killick-Kendrick R. Some epidemiological consequences of the evolutionary fit between leishmaniae and their phlebotomine vectors. *Bull Soc Path Ex*1985; 78:747-755. *PMID:3836763*
9. Schreiber GB, Busch MP, Kleinman SH, Koreiltz JJ. The risk of transfusion-transmitted viral infections. *New England J Med* 1996; 334:1685-9. *doi: 10.1056/NEJM199606273342601*
10. Marquez MF, Sanchez MJ, Sanchez AC, et al. *Leishmania infantum* (Protozoa, Kinetoplastida): transmission from infected patients to experimental animals under conditions that simulate needle-sharing. *Exp Parasitol* 2002; 100:71-4. *doi: 10.1006/expr.2001.4678*
11. Pagliano P, Carannante N, Rossi M. Visceral leishmaniasis in pregnancy: a case series and a systematic review of the literature. *J Antimicrob Chemother* 2005; 55:229-33. *doi: https://doi.org/10.1093/jac/dkh538*
12. Cruz I, Nieto J, Moreno J, et al. *Leishmania*/HIV co-infections in the second decade. *Ind J Med Res* 2006; 123:357-88. *doi: https://www.ncbi.nlm.nih.gov/pubmed/16778317*
13. Lainson R, Shaw, JJ. New World leishmaniasis the Neotropical *Leishmania* species: Topley & Wilson's microbiology and microbial infections, Parasitology, 9thedn. Vol. 5, ed. F.E.G Cox, J.P Kreier, D. Wakelin Oxford University Press (New York), 1998; 241-82.
14. Athukorale DN, Seneviratne JK, Ihalamulla RL, Premaratne UN. Locally acquired cutaneous leishmaniasis in Sri Lanka. *J Trop Med Hyg* 1992; 95:432–3 *PMID:1460704*
15. Siriwardana, HVYD, Chandrawans, PH, Sirimanna G, Karunaweera ND. Leishmaniasis in Sri Lanka: a decade old story. *Sri Lankan J Inf Dis* 2012; 2:2-12. *doi: http://dx.doi.org/10.4038/sljid.v2i2.4420*
16. Karunaweera ND, Abeysinghe R, Porter C, Dotson EM. Study on sandfly population in a cutaneous leishmaniasis focus in Sri Lanka. Proceedings of the 4th World Congress on leishmaniasis, India 2009; 323.
17. Abeygunasekara PH, Costa YJ, Seneviratne N, et al. Locally acquired visceral leishmaniasis in Sri Lanka. *Ceylon Med J* 2007; 52:30-1. *doi: https://doi.org/10.4038/cmj.v52i1.1047*
18. Rajapaksa US, Ihalamulla RL, Karunaweera ND. (2005) First report of mucosal tissue localization of leishmaniasis in Sri Lanka. *Ceylon Med J* 2005; 5:90-91. *PMID:16114778*
19. Siriwardana HV, Noyes HA, Beeching NJ, Chance ML, Karunaweera ND, Bates PA. *Leishmania donovani* and cutaneous leishmaniasis, Sri Lanka. *Emerg Infect Dis* 2007; 13:476–47. *doi: 10.3201/eid1303.060242*
20. Gajapathy K, Lalanthika BSP, Sara LG, et al. Molecular identification of potential leishmaniasis vector species within the *Phlebotomus (Euphlebotomus) argentipes* species complex in Sri Lanka. *Parasites & Vectors* 2013; 6:302. *doi: https://doi.org/10.1186/1756-3305-6-302*

21. Semage SN, Pathirana KPN, Agampodi SB. Cutaneous leishmaniasis in Mullaitivu, Sri Lanka: a missing endemic district in the leishmaniasis surveillance system. *Int J Infect Dis* 2014; 25:53–55. *doi: https://doi.org/10.1016/j.ijid.2014.03.1382*
22. Siriwardana HVYD, Thalagala N, Karunaweera ND. Clinical and epidemiological studies on the cutaneous leishmaniasis caused by *Leishmania (Leishmania) donovani* in Sri Lanka. *Annals Trop Med Parasitol* 2010; 104:213-22. *doi: https://doi.org/10.1179/136485910X12647085215615*
23. Sandanayaka R, Kahawita I, Gamage A, et al. Emergence of cutaneous leishmaniasis in Polonnaruwa, Sri Lanka 2008–2011. *Trop Med Int Health* 2014; 19:140–14. *doi :https://doi.org/10.1111/tmi.12232*
24. Galgamuwa LS, Sumanasena B, Yatawara L, et al. Clinico-Epidemiological patterns of cutaneous leishmaniasis patients attending the Anuradhapura Teaching Hospital, Sri Lanka. *Korean J of Parasitol* 2017; 55:1-7. *doi: 10.3347/kjp.2017.55.1.1*
25. Nawaratna SSK, Weilgama DJ, Wijekoon CJ, et al. Cutaneous Leishmaniasis, Sri Lanka. *Emerg Infect Dis* 2007; 13:1068–1070. *doi: 10.3347/kjp.2017.55.1.1*
26. Yatawara L, Wickramasinghe S, Rajapakse RPVJ, et al. Morphological and molecular studies on Sri Lankan *Leishmania*. *Trop Med Health* 2008; 36:171-179. *doi: https://doi.org/10.2149/tmh.2008-21*
27. Sharma NL, Mahajan VK, Kanga A, et al. Localized cutaneous leishmaniasis due to *Leishmania donovani* and *Leishmania tropica*: Preliminary findings of the study of 161 new cases from a new endemic focus in Himnchnl Pradesh in India. *Am J Trop Med Hyg* 2005; 72:819-2. *doi: https://doi.org/10.4269/ajtmh.2005.72.819*
28. Copeland HW, Arana BA, Navin TR. Comparison of active and passive case detection of cutaneous leishmaniasis in Guatemala. *Am J Trop Med Hyg* 1990; 43:257-9. *doi: https://doi.org/10.4269/ajtmh.1990.43.257*
29. Lainson R, Shaw, JJ. Evolution, classification and geographical distribution: The leishmaniases in biology and medicine. Academic Press, London 1987; p 1-12.
30. Daboul MD. Is the amastigote form of *Leishmania* the only form found in humans infected with cutaneous leishmaniasis? *Science* 2008; 39:39-41. *doi: https://doi.org/10.1309/F0LPBC27FEDQA7RQ*
31. Daboul MD. Application of the microscopic method in cutaneous *Leishmania* diagnosis. *Our Dermatology Online*.2011; 2:198-202.
32. Sharquie KE, Abdullah SH, Sabeeh AH, Ihssan A Al-H. Evaluation of diagnosis of cutaneous leishmaniasis by direct smear, culture and histopathology. *Saudi Med J* 2002; 23:925. *doi: https://www.ncbi.nlm.nih.gov/pubmed/12235464*
33. Ramírez RJ, Agudelo S, Muskus C, et al. Diagnosis of cutaneous leishmaniasis in Colombia: the sampling site within lesions influences the sensitivity of parasitologic diagnosis. *J Clin Microbiol* 2000; 38: 3768–377. *PMID: 11015400*
34. Gumurdulu D, Ergin M, Tuncer I, et al. Histopathological and clinical evaluation of the cutaneous leishmaniasis in Southern Anatolia, Turkey. *Aegean Pathology Journal* 2004; 1:57–61. *No doi*

35. Daboul MD. Toward an approach of cutaneous *Leishmania* treatment. *Our Dermatol Online. 2013;* 4(1):46-54 *doi: 10.7241/ourd.20131.09*

Research article

Molecular characterization and antibiotic sensitivity testing of bacteria in blood cultures of Hepatitis B virus infected subjects

II Anibijuwon[1], OC Ayanwale[1] and OO Ayanda[1]

Sri Lankan Journal of Infectious Diseases 2019 Vol.9 (1):68-78

DOI: http://dx.doi.org/10.4038/sljid.v9i1.8228

Abstract

Introduction: Hepatitis B virus is one of the most common infections worldwide. Many infected people are at risk of developing liver complications. Screening for common pathogenic bacterial infections that could contribute to complications is important for early diagnosis and appropriate management.

Methods: A cross sectional study was carried out on subjects aged 20-75 years for a period of 6 months (November 2016 to April 2017). Blood cultures and HBsAg rapid tests were performed on all 122 blood samples collected in Ilorin Metropolis. The screening was carried out on 92 HbsAg positive patients who presented with fever, and 30 apparently healthy HbsAg positive donors from the blood bank.

Results: Of 92 symptomatic patients, 44 (47.8%) had postive blood cultures and of the 30 HBV positive blood donors, 9 (30%) had positive blood cultures.

The prevalence of *Escherichia coli*, *Staphylococcus aureus*, P*seudomonas aeruginosa* and *Klebsiella pneumoniae* in Hepatitis B positive subjects was 5.7% (n=7), 5.7% (n=7), 23.8% (n=29) and 9.8% (n=12) respectively. In the apparently healthy HbsAg positive blood donor group, only 9 samples showed positive bacterial growth of *P. aeruginosa.*

All the bacterial isolates were resistant to amoxycillin-clavulanic acid, erythromycin, ceftriaxone, ceftazidime, cefuroxime, and ciprofloxacin. On PCR, *Nuc, Stx2, Pf* and *PSUE* genes were demostrated in *E. coli, S. aureus, P. aeruginosa* and *K. pneumoniae* respectively.

Conclusion: This study showed a high percentage (45.1%) of bacteraemia in HBV infection. Early screening and treatment of HBV infection and concomitant bacterial infection is recommended to prevent complications.

---

[1]*Public Health Laboratory Unit, Department of Microbiology, Faculty of Life Sciences, University of Ilorin, P.M.B 1515, Ilorin, Kwara State, Nigeria.*

Address for correspondence: Ibikunle Ibitayo Anibijuwon, Public Health Laboratory Unit, Department of Microbiology, Faculty of Life Sciences, University of Ilorin, P.M.B 1515, Ilorin, Kwara State, Nigeria Telephone: +2348036115296 Email: kunledoexploit@gmail.com; kunledoexploit@yahoo.com

https://orcid.org/0000-0003-0051-0396

Received 24 September 2018 and revised version accepted 2 March 2019

Keywords: Hepatitis B, Liver, Antimicrobial susceptibility, Blood culture

Introduction

Many important questions still remain unanswered for many pathogen combinations. Co-infected individuals may be in worse health than those with single infections[1] and may also pose the biggest risk of transmission to others.[2] It is important to note that hepatitis is an occasional feature of the clinical symptoms induced by many agents of both living and non-living origin.

Hepatitis B virus (HBV) infection is an infection of major public health significance, being the 10th leading cause of death globally. HBV infection accounts for 500,000 to 1.2 million deaths globally each year.[3] HBV infection can cause acute hepatitis, acute liver failure, chronic hepatitis, or can cause an asymptomatic infection and approximately 15-40% of infected patients will develop cirrhosis, liver failure or hepatocellular carcinoma.[4]

Numerous studies have suggested that the genetic constitution of the host is a critical factor in determining the outcomes of HBV infection. Despite the effectiveness of the current vaccination policy, the prevalence of Hepatitis B infection remains high, and the burden for health services is considerable.[5]

Blood stream infection by Gram negative bacteria is a common complication in patients with cirrhosis. Patients with cirrhosis and ascites showed a higher susceptibility to bacterial infections because of their inadequate defence mechanisms.[6] Very little is known about the correlation between HBV and different bacterial infections due to lack of diagnostic capability. The most common pathogenic agents, which enter the liver by vascular routes, are *E. coli, K. pneumoniae, Salmonella* Typhi, *Proteus vulgaris*, *Streptococcus spp,* and *Staphylococcus* spp, but anaerobes may also be present.[7] *S. aureus, P. aeruginosa,* and *K. pneumoniae* have been implicated in liver abscess.[8] *P. aeruginosa* is one of the most common causes of bacteraemia in liver transplant recipients. *K. pneumoniae* of high virulence (hv*KP*) has also been reported to lead to liver abscess in apparently healthy young adults.[9] Among cases of pyogenic liver abscesses, *Staphylococcus aureus* has been established as the leading cause in most series.[10] Uropathogenic *E. coli* (UPEC) has been reported to play a significant role in development of primary biliary cirrhosis (PBS).[6]

Methods

Collection of samples and data

The study protocol was reviewed by the University Ethics Review Board of the Faculty of Life Sciences, University of Ilorin and approval to carry out research was obtained from the Ethics Review Committee of the State Ministry of Health. All subjects gave written informed consent and were assured that all information was confidential. The target population (with or without fever) were confirmed as HBsAg positive at the Kwara State Civil Service Hospital, Specialist Hospital Sobi, General Hospital Ilorin and Blood Banks Ilorin, Nigeria from November 2016 to May 2017. They were above 18 years and the subjects' socio-demographic information were collected by administering a questionnaire.

A total of 122 HBsAg positive blood samples from patients attending Kwara State Civil Service Hospital, Sobi Specialist Hospital, Ilorin General Hospital, and the Blood Bank, Ilorin, Nigeria were confirmed between November 2016 – May 2017

Ninety-two (92) of the blood samples were collected from HBsAg positive patients of Kwara State Civil Service Hospital, Sobi Specialist Hospital and Ilorin General Hospital. Thirty blood samples (30) were collected from apparently healthy HBsAg positive donors from the blood bank.

Seven ml of venous blood was taken aseptically by needle and syringe with 2ml dispensed in 18ml of Thioglycollate medium and 5ml into a plain bottle. The samples in plain bottles were left at room temperature for 20-30 minutes to clot, centrifuged at 3000rpm for 5 minutes, and the resultant clear serum samples were packaged and transported to the laboratory in the Department of Microbiology, University of Ilorin for serological analysis of HBV using HBsAg and HBcAg (LifeSpan BioSciences, inc) rapid testing kits. All samples were tansported in a cold box at +2 °C to +8 °C and kept at -20 °C in the laboratory for 18-24 hours.

Assay procedure for concomitant bacteria

Thioglycollate media samples were incubated for 7 days with intermittent sub-culturing on Blood Agar at 37 °C for 24hrs and examined for growth. Positive blood cultures were identified and sub-cultured on Blood Agar, MacConkey, Salmonella-Shigella Agar and Mannitol Salt Agar. Colonial and cellular morphology of the culture plates were observed, and necessary biochemical tests were carried out.[11]

Antibiotics susceptibility test and ESBL screening

This was done according to Clinical Laboratory Standard Institute (CLSI) Guidelines.[12] Standard antimicrobial discs for Gram negative and Gram positive organisms was placed onto the surface of the inoculated agar plates accordingly and incubated for 24 hours. The zones of inhibition were measured and interpreted using the CLSI guidelines. ESBL screening was also performed by the disk synergy test.[13]

DNA extraction and amplification of genes

Crude method was used for DNA extraction of the bacterial isolates and polymerase chain reaction (PCR) was carried out to detect Universal genes *Nuc, Stx2, Pf* and *PSUE* in isolated *S. aureus, E.coli, Klebsiella spp. and Pseudomonas spp.* respectively using the primers and cycling parameters listed in Table 1. PCR amplification of genes was carried out for each gene singly using geneAmp PCR system 9700 thermal cycler (Applied Biosystems). All PCR assays were performed directly from bacterial suspensions obtained after rapid DNA extraction method. For the amplification master mix, an aliquot of 2µl of the bacterial suspension was added to 23µl of PCR mixture containing 50mM KCl, 10mM Tris-HCl (pH 8.6), 1.5mM $MgCl_2$, 5% glycerol, 0.08% NP-40, 0.05% Tween-20, 0.2mM of each deoxynucleoside triphosphate (dATP, dTTP, dGTP, and

dCTP), 10µM of respective primers and 25 units/ml of Taq DNA polymerase. All PCR assay runs incorporated a reagent control (without template DNA).

Table 1: Summary of the primers and PCR operating conditions

Gene	Primer Sequence 5'→ 3'	Size	Cycle	Cycling Parameters
Nuc	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	274Bp	35	94 °C–30s; 5 5°C–30s; 72 °C–1 min
Stx1	GTT ACG GGA AGG AAT CAG GGT AAA CGC GGA AGG AAT CAG GGT	300Bp	35	94 °C–30s; 60 °C–30s; 72 °C–1 min
Psue	AGCGTTCGTCCTGCACAAGT TCCACCATGCTCAGGGAGAT	98Bp	35	94 °C–30s; 58 °C–30s; 72 °C–1 min
Pf	ATT TGA AGA GGT TGC AAA CGA T TTC ACT CTG AAG TTT TCT TGT GTT C	615Bp	35	94 °C–30s; 57 °C–30s; 72 °C–1 min;

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using 0.7% agarose gel in 0.5X Tris borate EDTA buffer (44.5mM Tris borate and 1 Mm EDTA, pH 8.3). At the end of the run, the gel was transferred to a syngene gel documentation system (Syngene, UK) for agarose gel visualization using UV light. The DNA bands were then captured and visualized with a short wave ultraviolet trans illuminator and photographed using Syngene gel bio imaging system (UV trans-illuminator).

Results

Table 2 shows the distribution of bacterial isolates among the 122 samples collected. Twenty nine (23.8%) of *P. aeruginosa*, 12 (9.8%) of *K. pneumoniae*, 7 (5.7%) of *S. aureus*, 7 (5.7%) of *E. coli* made up the 55 (45.1%) samples positive for bacterial infections. The prevalence of bacterial infections in patients with HBsAg was determined as 45.1%.

Table 2: Age distribution of the prevalence of bacterial isolates among all HBV +ve subjects

Age (years)	No Examined	Positive samples	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*S. aureus*
18-27	40	15	1	7	4	3
28-37	19	11	2	7	1	1
38-47	34	17	3	8	5	1
48-57	18	10	1	6	2	1
58-67	8	2	0	1	0	1
>68	3	0	0	0	0	0
Total	**122 (100%)**	**55 (45.1%)**	**7 (5.7%)**	**29 (23.8%)**	**12 (9.8%)**	**7 (5.7%)**

Table 3: Age distribution of the prevalence of bacterial isolates among HBsAg +ve patients

Age (years)	No Examined	Positive samples	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*S. aureus*
18-27	32	13	1	5	4	3
28-37	14	9	2	5	1	1
38-47	27	13	3	6	4	0
48-57	12	8	1	4	2	1
58-67	5	1	0	0	0	1
>68	2	0	0	0	0	0
Total	**92 (100%)**	**44 (47.8%)**	**7 (7.6%)**	**20 (21.7%)**	**11 (12.0%)**	**6 (6.5%)**

Table 4: Age distribution of the prevalence of bacterial isolates in healthy HBsAg +ve blood donors

Age (years)	No Examined	Positive samples	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*S. aureus*
18-27	8	2	0	2	0	0
28-37	5	2	0	2	0	0
38-47	7	2	0	2	0	0
48-57	6	2	0	2	0	0
58-67	3	1	0	1	0	0
>68	1	0	0	0	0	0
Total	**30 (100%)**	**9 (30.0%)**	**0 (0.0%)**	**9 (30.0%)**	**0 (0.0%)**	**0 (0.0%)**

Forty-five males (36.9%) and seventy-seven females (63.1%) were enrolled in the study. Table 5 shows the gender distribution of the bacterial isolates

Table 5: Gender distribution of the prevalence of bacterial isolates among HBV +ve subjects

Gender	Samples	*E.coli*		*S.aureus*		*P.aeruginosa*		*K. pneumonia*	
		n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Male	45	3	6.7	4	8.9	13	28.9	3	6.7%
Female	77	4	8.9	3	3.9	16	6.67	9	6.6
Total	**122**	**7**		**7**		**29**		**12**	

Figure 1 compares the distribution of bacterial isolates in the HBV positive patient group and HBV positive control group (blood donors). *P. aeruginosa* was the only isolate in the blood donor group.

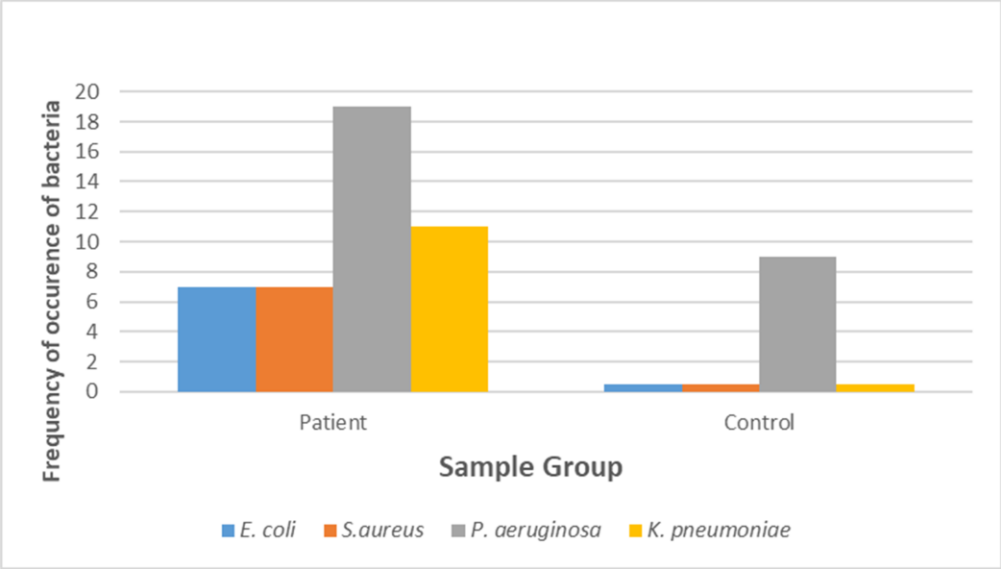


Figure 1: Distribution and comparison of bacterial isolates in HBV positive subjects (patients and control)

Antibiotic susceptibility testing of the *S. aureus* isolates showed multiple resistance to amoxycillin-clavulanic acid (100%), erythromicin (100%), cloxacillicin (100%), and ciprofloxacin (100%). High sensitivity was also recorded with ofloxacin (85.7%), and gentamicin (71.4%).

As shown in Table 5, antibiotic susceptibility testing for the Gram negative bacteria shows high resistance to ceftazidime (100%), cefuroxime (100%), ciprofloxacin (100%), amoxycillin-clavulanic acid (97.9%), and ceftriaxone(91.6%). *E. coli* and *K. pneumoniae* showed high level susceptibility to ofloxacin (83.3%), imipenem (83.3%), gentamicin (75%).

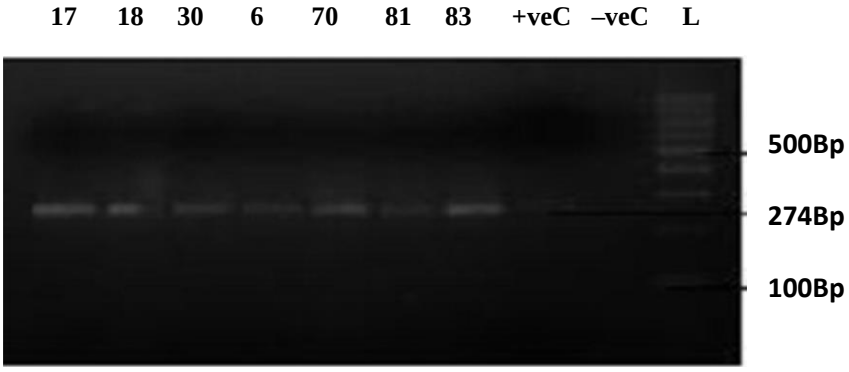
Phenotypic detection of extended spectrum β lactamase (ESBL) production showed that 7 (24.1%), 3 (50%) and 3 (25%) of the *P. aeruginosa*, *E. coli* and *K. pneumoniae* isolates were ESBL producers.

Table 6: Antibiotic susceptibility of Gram negative isolates from patients

Antibiotic	*Pseudomonas aeruginosa* n=29				*Klebsiella pneumoniae* n=12				*Escherichia coli* n=7			
	S	%	R	%	S	%	R	%	S	%	R	%
Augmentin (30µg)	Not tested				0	0	12	100	0	0	7	100
Ceftriaxone (30µg)	Not tested				0	0	12	100	0	0	7	100
Ceftazidime (30µg)	0	0	29	100	0	0	12	100	0	0	7	100
Cefuroxime (30µg)	0	0	29	100	5	41.6	7	58.3	5	71.4	2	28.6
Gentamicin (10µg)	23	80	6	20	0	0	12	100	0	0	7	100
Ciprofloxacin (10µg)	0	0	29	100	0	0	12	100	0	0	7	100
Imipenem (10µg)	27	93.1	2	6..9	4	33.3	8	66.7	4	57.1	3	42.9
ESBL positive	22	75.9	7	24.1	9	75	3	25	4	57.1	3	42.9

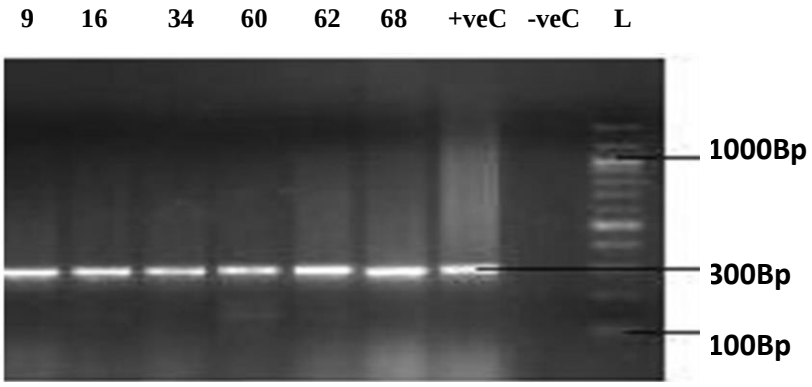
S-sensitive, R-resistance

Figures 2-5 demonstrate the relevant genes in the four species isolated from patients in the study.



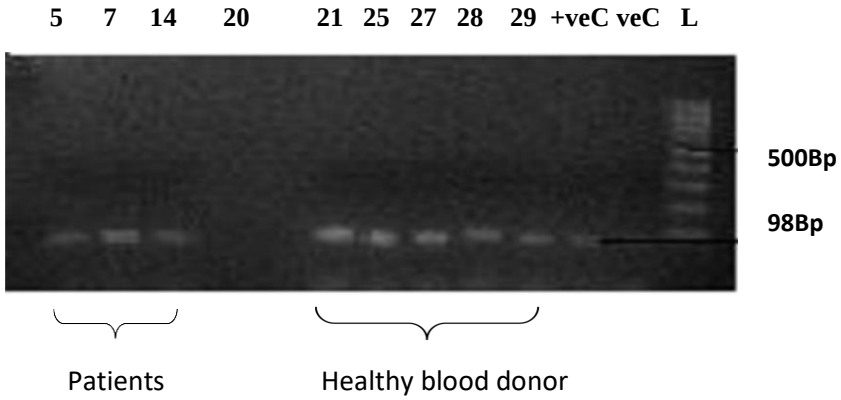
+ve- C positive control; –veC- negative control; L - Ladder

Figure 2: PCR confirmed *NUC* gene in *S. aureus* isolates



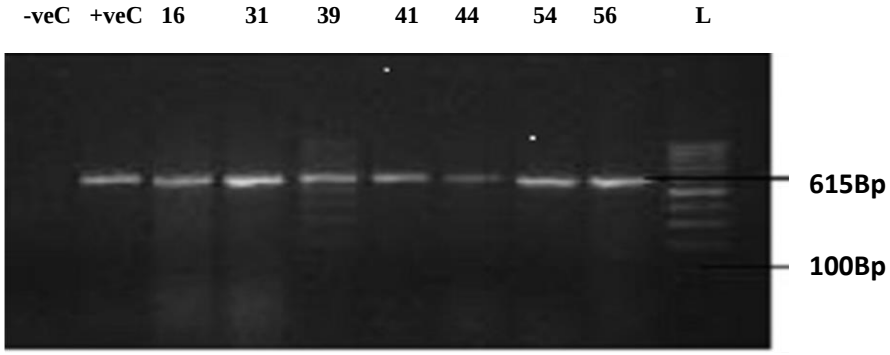
+ve C – positive control: -veC – negative control; L – ladder

Figure 3: PCR confirmed *Stx2* gene in *Escherichia coli* isolates



+ve C – positive control: -veC – negative control; L – ladder

Figure 4: PCR confirmed *PSUE* gene in *P. aeruginosa* isolates.



+ve- C positive control; –veC- negative control; L - Ladder

Figure 5: PCR confirmed *Pf* gene in *Klebsiella pneumoniae* isolates

Discussion

The severity of liver infection is likely to contribute to susceptibility to co-infection and bacterial infections can also contribute to the chronicity of disease in HBV patients. Bacterial infections are common in patients suffering with viral hepatitis and are critical for prognosis.[14] Other studies also have confirmed the association between bacterial infections and HBV and also bacterial infections in cirrhotic patients as an important cause of morbidity and mortality.[15]

It is important to note that most of the patients in the current study were in their acute stage although liver function tests were not determined. In this study, the prevalence of bacterial infections in patients with HBsAg was determined as 45.1%.

The organisms isolated were *P. aeruginosa*, *E. coli*, *K. pneumoniae*, and *S. aureus*. *P. aeruginosa* was isolated most often in both patients and blood donors in this study which was similar to a study conducted in Korea.[16] Sample cultures with no bacterial growth were 67 (54.9%) and *Salmonella* Typhi was not isolated in this study. *S. aureus* and *E. coli* were each isolated in 5.7% of the samples, in contrast with a similar study which reported 43.2% for *S. aureus*[15] and 54.5%[12] for *E. coli*. *K. pneumoniae* reported in this study was 9.8%, which was similar to a study conducted in China that reported approximately 20%.[14]

All *K. pneumoniae* isolates in the current study were resistant to ciprofloxacin as also reported by Alo et al[17] and Cheong et al[15] who reported 80% and 100% resistance respectively. It is notable that bacteraemia was significantly associated with the presence of biliary disease in cirrhotic patients and one of the most implicated bacteria was *P.aeruginosa*.[17] In this study, *P. aeruginosa* demonstrated very high resistance to ciprofloxacin and ceftazidime similar to other studies.[18] The ceftazidime resistance in this study was high (100%), in agreement with 91% that was reported in Egypt.[18]

Antibiotic susceptibility of *E. coli* isolates in the current study were similar to those previously reported. Kibret et al reported *E. coli* resistant to ciprofloxacin (79.6%) and gentamicin (71.4%) in a study in Ethiopia.[19] All *E. coli* isolates in the current study were resistant to ciprofloxacin, similar to the results of Cheong et al.[15] Ciprofloxacin resistance of *S. aureus* in the current study was 100%, similar to a study conducted in Korea which demonstrated very high resistance to ciprofloxacin and oxacillin.[11]

Of the *E. coli* isolated in the current study, 42.9% were ESBL producers. Similar results have been reported from Pakistan (56.9%)[20] and India (40%).[13]

Of *K. pneumoniae* isolated in the current study, 25% were ESBL producers. A similar result (33%) was obtained in a study in Iran[21] in contrast to an African study which reported 71.7%.[22]

Of *P. aeruginosa* isolated in the current study, 24%were ESBL producers. These results were similar to other studies which reported 22%[11] and 13.79%[23] ESBL production. The ESBL producing *P. aeruginosa* isolates exhibited co-resistance against most of the antibiotics tested, consistent with results of other recent studies.[8,18]

The high incidence of ESBL producers among the isolates in the current study has considerable health implications as shown in recent studies, where infection with ESBL producing Gram negative bacilli resulted in significantly higher fatality rates than those with non-ESBL isolates.[8]

Conclusion

Bacteraemia with or without symptoms appear to occur in those demonstrated to be HBV positive. Further studies to follow up such patients and assess the significance of these bacteraemic episodes would be useful, particularly as a significant number of the isolated organisms showed antibiotic resistance to commonly used antibiotics.

REFERENCE

1. Griffiths EC. The nature and consequences of coinfection in humans. *J. Infect.*2014; 63:200–206. *doi: http://dx.doi.org/10.1016/j.jinf.2011.06.005*
2. Lass S. Generating super-shedders: co-infection increases bacterial load and egg production of a gastrointestinal helminth. *J. R. Soc.Interface.*2013; 10:20120588. *doi: http://dx.doi.org/10.1098/rsif.2012.0588*
3. Gonzalez SA, Keeffe EB. Risk assessment for hepatocellular carcinoma in chronic hepatitis B: scores and surveillance. *International Journal of Clinical Practices* 2012; 66:7–10. *doi: http://dx.doi.org/10.1111/j.1742-1241.2011.02808*
4. Takako I, Yasuhito T. Hepatitis B virus and its sexually transmitted infection – an update. *Microbial Cell* 2016; 3(9):420-437. *doi:10.15698/mic2016.09.527*
5. Zoulim F, Locarnini S. Optimal management of chronic hepatitis B patients with treatment failure and antiviral drug resistance. *Liver International Supply* 2013; 1:116–124 *doi: .http://dx.doi.org/10.1111/liv.12069*
6. Koutsoumpas AL, Smyk DS, Bogdanos DP. *E. coli*induced experimental model of primary biliary cirrhosis: At last. *International Journal of Hepatology* 2014; 1-6. *doi:http://dx.doi.org/10.1155/2014/848373*

7. Petri A, Tiszlavicz L, Nagy E. Liver abscess caused by *Stenotrophomonas maltophilia*: Report of a case. *Surg Today* 2003; 33(3):224-8. *doi: http://dx.doi.org/10.1007/s005950300050*
8. Yaita K. Liver abscess caused by multidrug-resistant *Pseudomonas aeruginosa* treated with colistin; a case report and review of the literature. *International Medicine* 2013; 52 (12):1407-1412. *doi:http://dx.doi.org/10.2169/internalmedicine.52.9296*
9. Lin D, Koskella B. (2014). Friend and foe: factors influencing the movement of the bacterium *Helicobacter pylori* along the parasitism–mutualism continuum. *Evol. Appl.*2014; 8:9–22. *doi: http://dx.doi.org/10.1111/eva.12231*
10. Reddy PN, Srirama K, Dirisala VR. An Update on Clinical Burden, Diagnostic Tools, and Therapeutic Options of *Staphylococcus aureus. Infect Dis (Auckl)* 2017; 10: *doi: 10.1177/1179916117703999*
11. Park JK, Lee CJ, Kim IH, et al. Clinical Characteristics and Prognostic Impact of Bacterial Infection in Hospitalized Patients with Alcoholic Liver Disease**.** *J Korean Med Sci.* 2015; 30 (5):598-605. *doi:https://doi.org/10.3346/jkms.2015.30.5.598*
12. CLSI (Clinical and Laboratory Standard Institute). Performance Standards for Antimicrobial Disc Susceptibility Tests; Approved Standard—Eleventh Edition. CLSI document M02-A11, Wayne, PA, USA., 2012.
13. Kumar D, Singh AK, Ali MR, et al. Antimicrobial Susceptibility Profile of Extended Spectrum β-Lactamase (ESBL) Producing *Escherichia coli* from Various Clinical Samples. *Infect Dis (Auckl)* 2014; 7: 1–8. *doi: 10.4137/IDRT.S13820*
14. Wei Li, RonghuaJin, Peng Chen, Guoxian Zhao, Ning Li &Hao W Clinical correlation between HBV infection and concomitant bacterial infections. *Sci. Rep* 2015; 5, 15413; *doi: https://doi: 10.1038/srep15413*
15. Cheong HS, Kang CI, Lee JA. Clinical significance and outcome of nosocomial acquisition of spontaneous bacterial peritonitis in patients with liver cirrhosis. *Clin Infect Dis*2014; 48:1230–1236. *doi:https://doi.org/10.1086/597585*
16. Tian LT, Yao K, Zhang XY, et al. Liver abscesses in adult patients with and without diabetes mellitus: an analysis of the clinical characteristics, features of the causative pathogens, outcomes and predictors of fatality: a report based on a large population, retrospective study in China. *Clin Microbiol Infect* 2012; 18: E314–E330. *doi: https://doi.org/10.1111/j.1469-0691.2012.03912*
17. Alo MN, Anyim C, Igwe JC, et al. Presence of extended spectrum β-lactamase (ESBL) *E. coli* and *K. pneumonia* isolated from blood cultures of hospitalized patients. *Advances in Applied Science Research* 2012; 3(2):821-825 *No doi*
18. Mahmoud BA, Zahran WA, Hindawi GR, et al. Prevalence of multidrug-resistant *Pseudomonas aeruginosa* in patients with nosocomial infections at a University Hospital in Egypt, with special reference to typing Methods. *Journal of Virology &Microbiology* Vol. 2013, 1-13. *doi: https//doi.org/ 10.5171/2013.29004*
19. Kibret M, Abera B. Antimicrobial susceptibility patterns of *E. coli* from clinical sources in northeast Ethiopia. *Afric Health Sci* 2011; 11:S40–S45 *No doi*
20. Ullah F, Malik SA, Ahmed J. Antibiotic susceptibility pattern and ESBL prevalence in nosocomial *Escherichia coli* from urinary tract infections in Pakistan. *Afr. J. Biotechnol* 2009; 8(16):3921–3926 *No doi*
21. Ghafourian S, Sekawi ZB, Sadeghifard N, et al. The Prevalence of ESBLs Producing *Klebsiella pneumoniae* Isolates in Some Major Hospitals, Iran. *Open Microbiol J* 2011; 5: 91–95. *doi: 10.2174/1874285801105010091*
22. Ejaz I, Aizza Z, SaqibM, et al. Urinary tract infections caused by extended spectrum β-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumonia. African Journal of Biotechnology* 2011; 10(73):16661-16666, *doi: 10.5897/AJB11.2449 ISSN 1684–5315 © Academic Journals*
23. Shaikh S, Fatima J, Shakil S, et al. Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and Treatment. *Saudi Journal of Biological Sciences* 2015; 22:90–101. *doi:https://doi.org/10.1016/j.sjbs.2014.08.002*

Research article

A preliminary survey of knowledge, attitudes and practices regarding rabies in West Bengal, India

Subhabrata Kumar[1]

Sri Lankan Journal of Infectious Diseases 2019 Vol.9 (1):79-83

DOI: http://dx.doi.org/10.4038/sljid.v9i1.8233

Abstract

Introduction: This study was conducted to evaluate the knowledge, attitudes and practices (KAP) of patients attending the general outpatient department in Malda Medical College about rabies.

Methods: A structured questionnaire was answered by 161 participants. The KAP score was calculated according to the response of the participant.

Results: Compared to adults, children in the 10-15year age group scored much less in all components of rabies prevention and post exposure management.

Conclusion: There was lack of awareness about post exposure prophylaxis of rabies in children. Awareness campaigns especially focusing on children are required to provide better medical care.

Keywords: *Rabies, Education, Children, Medical care*

Introduction

Annual deaths caused by rabies in India during 2001-2003 is shown in Table 1.[1] The Central Bureau of Health Intelligence, Government of India, has published that in the year 2016 a total of eighty six people died due to rabies in India. In West Bengal, forty seven people died due to rabies in the same year.[2] Malda is located near the Bangladesh border in West Bengal, India.

Most human deaths due to rabies are dog mediated. Human deaths due to rabies are largely seen in rural areas in communities with poor socio-economic status. The main reason for human deaths is lack of awareness among people about the importance of post-exposure prophylaxis. Rabies is a nearly hundred percent vaccine preventable disease. Awareness about rabies and anti-rabies vaccine can make a significant contribution in eliminating rabies.[3]

[1]*Address for correspondence: Dr Subhabrata Kumar, Assistant Professor, Department of Pharmacology, Malda Medical College and Hospital, West Bengal, India Pin-732101 Telephone +918670095183*

Email: subhabrata.kumar@gmail.com *https://orcid.org/0000-0002-3619-3524*

Received 23 October 2018 and revised version accepted 22 March 2019

Table 1: Annual deaths due to rabies in India (2001-2003)[1]

Age in years	Proportion of rabies deaths/ 1000 deaths
0-4	1.1
5-14	11.5
15-29	1.6
30-44	1.9
45-59	1.4
60-69	0.5
70+	0.3
All ages	1.3

This study has been undertaken to evaluate the knowledge, attitudes and practices of a sample population attending the Outdoor Patient Department of Malda Medical College and Hospital, Government of West Bengal. The study findings can assist in identifying areas which need to be addressed by future health education programs.

Methods

Study area

This survey was conducted from April 2017 to January 2018 at the General Outdoor Patient Department (GOPD) of Malda Medical College & Hospital, West Bengal in India.

Sample size

Participants were recruited according to the convenient sampling method. One hundred and sixty one patients who attended the GOPD for various health problems were recruited to the study. Participation was voluntary, and the collected data was kept confidential.

Study questionnaire design

The questionnaire consisted of both open and close-ended questions. Interviewer assisted data collection was done in the participants' native language of Bengali to give a better chance of understanding the content of the question. The questions focused on collecting information about knowledge, attitude and practices with regard to rabies. The questionnaire was pre-tested.

Questions regarding the knowledge component covered the description of clinical rabies, mode of transmission, outcome, varieties of animals affected by rabies, prevention and control.

Questions related to attitudes covered the outlook towards wound washing, and attending a hospital following exposure.

The practice component included questions on management of a dog bite (washing the wound, going to hospital, taking anti-rabies vaccine, taking tetanus toxoid, taking antibiotics), and observed practice(s) towards a suspect rabid animal or a carcass of a suspect rabid animal.

Scoring system

Scores were given according to the accuracy and completeness of response by the participants. Scores ranged from zero to two as per nature of the question. Scoring on the knowledge component of post-exposure prophylaxis when exposed to a dog bite is given in Table 2.

Table 2 Example of the scoring system

	Marks
Post exposure prophylaxis known	2
Post exposure prophylaxis unknown Antibiotics and anti-tetanus treatment known	1
Knowledge about treatment unknown	0

A score of 60% or above was considered good for all 3 categories of knowledge, attitudes and practices. The combined score - the KAP score – was calculated by simple addition of the scores from the three components. A KAP score above 60% was considered to be a good KAP score.

Data collection

Participants aged 10 years and above were recruited for the study. The questions were read out to the participants and the responses were noted down securely.

Data analysis

Odds ratio was calculated and p value < 0.05 was considered to be statistically significant. The data was analysed using online statistical data analysis software (https://www.medcalc.org)

Results

A total of 161 participants answered the questions. Of the 161, 26% obtained a KAP score of >60% (considered as good) and 74% had a KAP score of less than 60% (considered as a poor score).

Socio-demographic characteristics

Participants resided in the rural areas. The majority of participants belonged to the poor socio-economic group. Of the 161, 61% were Muslims and 39% Hindus.

The age distribution of the participants is given in Table 3. Majority (96%) of the participants were males.

Table 3
Age distribution of participants

Age in years	%
10-15	7
16-30	42
31-50	31
>51	20

The distribution of knowledge about rabies is shown in Table 4 and that on attitudes and practices in Table 5.

Table 4: Knowledge about rabies

Knowledge component	Yes	No
Knows about rabies as a disease	42	119
Rabies is transmitted by dogs	144	17
Rabies is transmitted by other animals	127	34
Rabies is always fatal	106	55

As shown in Table 4, most of the participants knew that rabies spreads through a dog bite (n=144). Although the majority knew that rabies was invariably fatal, 55 of the 161 participants were not aware of this fact.

There was a significant association of the management of dog bite score with age (Odds ratio: 0.0648, 95% confidence interval: 0.0081-0.5198, Z statistics: 2.576, significance level p=0.0100). The result is significant at p<0.05. Children (10-15 years) scored significantly

less as compared to participants older than 15 years in management of dog bite (Table 6).

Table 5: Attitudes and practices towards rabies

Component	Yes	No
Hospital presentation and post exposure prophylaxis following a dog bite.	92	69
Get the suspect rabid animal killed	125	36

Table 6: Comparison between children (10-15 years) and participants-older than 15 years in management of dog bite

	Good	Poor
10-15 years	1	10
Participants older than15 years	91	59

Discussion

Rabies is a significant health burden in India.[2] Dog mediated transmission is the most common cause of human rabies which was known by 144 of the 161 participants. Rabies is always fatal once symptoms of the disease start to occur and the majority of the participants (n=106) knew this. However, rabies is a preventable disease. A large number of clinical studies have been conducted worldwide to highlight this key aspect.[4,5,6,7,8]

Global statistics have shown that 90% of human deaths due to rabies occur in children. Deaths in children occur because they like playing with dogs and lack awareness about transmission of rabies by dogs. Children also try to hide dog bites and scratches for fear they might be reprimanded by their parents. To achieve the Rabies ZERO by 2030 target, the Ministry of Health and Family Welfare, Government of India has implemented the National Rabies Control Programme. One of the important aspects stressed in this programme was the education of children to prevent dog bites and encouraging parents to praise a child who tells them about a dog bite or scratches. The guidelines also asked people to avoid treating the wounds with indigenous products like turmeric, soil etc. Importance of immediate medical care after a dog bite was advocated throughout the National Rabies Control Programme.[10]

Studies conducted in school children have shown improvements in knowledge, attitudes and practices towards rabies management among them.[11,12] This study conducted in Malda District, West Bengal can improve the awareness about rabies in children and provide better medical care.

Limitation of this study: This study showed that as far as knowledge about post exposure prophylaxis of rabies is concerned, children below 15 years scored significantly less as compared to other age groups. However, the number of children who participated in the current study was limited. A study involving a larger number of children is required to confirm these results.

Conclusion

There was lack of awareness about post exposure prophylaxis of rabies in children as compared to older age groups. As lack of knowledge and awareness contributes to continuing exposure to rabies, we recommend an intentional programme to sensitize the population about the risk factors for rabies and post exposure management. The use of electronic media and other methods to specifically target children is indicated if the results of our study are confirmed in a larger study.

Acknowledgement: All the participants, my colleagues and my family members

Conflict of Interest: Nil

Ethical statement: The study was approved by the Institutional Ethics Committee, Malda Medical College, Malda, Government of West Bengal.

References:

1. Suraweera W, Morris SK, Kumar R, et al. Deaths from symptomatically identifiable furious rabies in India: A nationally representative mortality survey. *PLoS Negl Trop Dis* 2012 6(10): e1847. *doi:10.1371/journal.pntd.0001847*
2. Central Bureau of Health Investigation, Government of India. National Health Profile 2017 Part -1. State/UT wise Cases and deaths due to rabies in India, 2016. *http://cbhidghs.nic.in* - accessed March 2018
3. World Health Organization: Rabies Home Page on internet. Available from: *www.who/int/rabies/en*.accessed March 2018
4. JT Muthunuwan, AGKH Ganhewa, HDSG Perera *et al.* Preliminary survey on knowledge, attitude and practice regarding rabies. *Sri Lankan Journal of Infectious diseases* 2017; 7(1):38-46 *doi: http://doi.org/10.4038/sljid.v7i1.8133*
5. Gino C. Matibag, Yoshihide Ohbayashi, Koji Kanda, et al. A pilot study on the usefulness of information and education campaign materials in enhancing the knowledge, attitude and practice on rabies in rural Sri Lanka. *J Infect Developing Countries* 2009; 3(1):55-64. *doi:https://doi.org/10.3855/jidc.106*
6. Sambo M, Lembo T, Cleaveland S et al. Knowledge, Attitudes and Practices (KAP) about rabies prevention and control: A community survey in Tanzania. *PLoS Negl Trop Dis*. 2014 ;8(12): e3310.*doi:10.1371/journal.pntd.0003310*
7. Tripathy RM, Satapathy SP, Karmee N. Assessment of knowledge, attitude and practice regarding rabies and its prevention among construction workers: a cross-sectional study in Berhampur, Odisha. *Int J Res Med Sci* 2017;5:3970-5. *doi: http://dx.doi.org/10.18203/2320-6012.ijrms20173964*
8. Suhasani Tandon, S.K. Kotwal, M.A. Malik et al. A community based survey on rabies control and prevention using KAP in Jammu, India. *Journal of Animal Research.* 2017;7(6):1019-1028. *doi: http://dx.doi.org/10.5958/2277-940X.2017.00153.X*
9. Medcalc Software. Available from https://www.medcalc.org [Assessed on March 2019]
10. National Health Portal, India . Available at *www.nhp.gov.in/world-rabies-day.* Accessed on March 2018
11. Hossain M. Study on knowledge, attitude &practice about rabies &pet animals among school children in Bangladesh. *J Microbiol Exp* 2017; 4(1):00103 *doi: 10.15406/jman.2017.04.00103*
12. Burdon Bailey JL, Gamble L, Gibson AD et al.A rabies lesson improves rabies knowledge amongst primary school children in Zomba, Malawi. *PLoS Negl Trop Dis*2018: 12(3):e0006293. *doi:https://doi.org/10.1371/journal.pntd.0006293*

Case Report

Intra-familial transmission of hepatitis B affecting all household members A case report

MIP Premathilake[1], RGLS Rajamanthree[1], YLB Karunarathne[1], MARV Muthugala[1]

Sri Lankan Journal of Infectious Diseases 2019 Vol.9 (1):84-87

DOI: http://dx.doi.org/10.4038/sljid.v9i1.8235

Abstract

Intra-familial transmission of hepatitis B is well documented and is the rationale for screening of household members. However, reports on transmission of infection to all household members are sparse. We report a case of intra-familial transmission of hepatitis B affecting all household members. The index case was a lady diagnosed with chronic liver cell disease, who was later found to have chronic hepatitis B viral infection. All household contacts were screened, which included five persons. All showed evidence of exposure and two were chronically infected, of which one was a pregnant lady. The risk of familial transmission of hepatitis B could be higher than expected. This case highlights the importance of active efforts to screen all family members at diagnosis of each new case of hepatitis B.

Keywords: Hepatitis, Sri Lanka, Familial, Transmission, Infection

Introduction

Hepatitis B is a partially double stranded DNA virus belonging to the family *Hepadnaviridae*. It is a leading cause of acute and chronic hepatitis in the world. However, in contrast to the regional epidemiology, Sri Lanka is considered to be a low-prevalent country for the infection.[1] The virus is transmitted through blood and blood products, percutaneous inoculation, sexual exposures, mother to child and horizontally through chronic exposure to infectious saliva.[2]

Diagnosis of hepatitis B infection is denoted by the presence of hepatitis B surface antigen (HBsAg).[3] Chronic infection is determined by the presence of HBsAg for more than six months.[3] Exposure to natural infection is determined by the presence of hepatitis B core antibodies (HBcAb). Presence of Hepatitis B envelope antigen (HBeAg) indicates a high replicative state and seroconversion to antiHBe reflects low infectivity.[3]

[1]*Teaching Hospital, Kandy, Sri Lanka*

Address for correspondence: Dr Ishara Premathilake, 427, Old Kottawa Road, Udahamulla, Nugegoda, Sri Lanka

Telephone: +94 0776010964 Email: ipremathilake@yahoo.com https://orcid.org/0000-0002-0083-6145

Received 21 November 2018 and revised version accepted 17 March 2019

Current recommendation for contact screening for hepatitis B infection is by testing for both HBsAg and HBcAb, or by initial testing for HBcAb, followed by HBsAg for all positive contacts.[3] Although hepatitis B transmission could be as high as 11-57% among household contacts,[4,5] infection of all family members in a single household is uncommon. We report a case of hepatitis B infection which occurred among all household members during routine contact screening of an index case.

Case Report

The index patient, a 53 year old female presented to the Teaching Hospital, Kandy with bilateral ankle swelling and abdominal distention of 1 week duration. She was diagnosed with chronic liver cell disease with a Child and Pugh score C and MELD (Model for End-stage Liver Disease) B. She was positive for HBsAg, HBcAb and HBeAg without evidence of HBeAb. Her initial viral DNA load was 3.2×10^3 IU/ml. Alanine transferase was elevated more than ten times the upper limit of normal. The patient was managed with a liver failure regimen and was started on the antiviral, tenofovir disoproxil fumarate. The family was referred to the Department of Virology for screening.

The household members included the patient's husband, three daughters and an elder sister who had been living in the same house for three years.

All household members were screened for both HBsAg and HBcAb. All were positive for HBcAb with two having detectable HBsAg. The contacts who were HBsAg positive were the patient's eldest daughter and sister (Figure 1). The contacts who were HBsAg negative had immunity against the infection with HBsAb levels >400IU/ml and no further action was taken. They were educated about the past infection and the need to reveal this information at healthcare checks.

Both newly diagnosed HBsAg positive patients were negative for HBeAg and positive for HBeAb.

The patient's eldest daughter who was newly diagnosed, was married and undergoing assisted reproductive treatment (ART) by the time she was diagnosed. Her husband was negative for all markers and an accelerated hepatitis B immunization schedule was commenced for him. It was suggested to postpone pregnancy until her hepatitis B status was fully evaluated and controlled. However, she became pregnant before any further investigations/management could be done. Her hepatitis B DNA was undetectable, and it was planned to repeat the test at mid-trimester. She was directed to the care of a gastroenterologist.

The index patient's sister's viral load was found to be 5.1×10^3 IU/ml. She was asymptomatic at the time of diagnosis and was referred to the gastroenterologist for further management.

Discussion

Intra-familial transmission of hepatitis B is an important infection control issue. All healthcare providers need to play an active role in encouraging contacts to be screened. In this instance all household members had acquired the infection by the time of presentation of the index case.

It is possible that both the index patient and her sister acquired the infection congenitally as both of them were chronically infected. Congenital hepatitis B can cause chronic infection in up to 90% of patients.[2] We could not pursue this any further as both parents and all other siblings were not alive. As all children of the index patient were infected it is possible that they too acquired the infection perinatally. It is also possible that some family members acquired the infection through horizontal transmission, especially the two younger daughters, who did not have detectable HBsAg. Although horizontal transmission is well described in families,[6] it is primarily observed among children in hyperendemic areas.[7,8]

It is demonstrated that only <5% will develop chronicity if the infection is acquired during adulthood compared to 80-90% in perinatal infections.[2] Those patients who clear the HBsAg will develop protective immunity as in the case of these contacts. This is also true for the husband who had probably acquired the infection through sexual transmission.

Management of hepatitis B in pregnancy plays an important role in prevention of mother to child transmission. Current guidelines recommend administration of Hepatitis B vaccine to newborns of HBsAg positive mothers with or without Hepatitis B immunoglobulin, depending on the infectivity of the mother, within 12 hours of birth.[2,7,9] Antiviral therapy in the second trimester is recommended for mothers with DNA load>200,000 IU/mL even if they do not meet the routine treatment indications, as a measure to prevent mother-to-child transmission.[3] The pregnant mother in this case was detected in very early stages of her precious pregnancy and she was linked to the relevant care team for further management.

We also detected the patient's sister as a new case of chronic hepatitis. Knowing the hepatitis B exposure status will be important to other contacts as well, especially as there is a possibility of reactivation of the virus during cancer chemotherapy or immunosuppression as the virus is never completely cleared from the body.

The strength of this case was that it was possible to screen all household members of the index case. This case could have been further benefited by genetic sequencing and phylogenetic analysis in proving the routes of transmission and to determine any enhanced infectivity of the virus.

Conclusion

Transmission dynamics among household contacts is complex. Screening all contacts of the index patient is an essential part of infection control in the community and individual patient management as illustrated by this case.

Acknowledgement

We thank the patient and the family for consenting to publication of the case details.
We would also like to thank the gastroenterology team at Teaching Hospital Kandy for the clinical information and follow up of patients.

Conflict of interest: None declared

References

1. Noordeen F, Pitchai FNN, Rafeek RA. A review of hepatitis B virus infection in Sri Lanka. *Sri Lankan Journal of Infectious Diseases* 2015; 5 (2):42-50. *doi: https://dx.doi.org/10.4038/sljid.v5i2.8087*
2. World Health Organization. Hepatitis B vaccines: WHO position paper - recommendations. *Vaccine*. 2010;28(3):589-90. *doi: https://doi.org/10.1016/j.vaccine.2009.10.110*
3. Terrault NA, Lok ASF, McMahon BJ, et al. Update on prevention, diagnosis, and treatment of chronic hepatitis B: AASLD 2018 Hepatitis B Guidance *Hepatology* 2018;67(4):1560-1599. *doi: https://doi.org/10.1002/hep.29800*
4. Lobato C, Tavares-Neto J, Rios-Leite M, et al. Intrafamilial prevalence of hepatitis B virus in Western Brazilian Amazon region: epidemiologic and biomolecular study. *J Gastroenterol Hepatol*. 2006;21(5):863-8. *doi*: *https://dx.doi.org/10.1111/j.1440-1746.2006.04298.x*
5. Alizadeh MAH, Ranjbar M, Ansari S, et al. Intra-familial prevalence of hepatitis B virologic markers in HBsAg positive family members in Nahavand, Iran. *World J Gastroenterol*. 2005;11(31):4857-60 *doi: https://dx.doi.org/10.3748/wjg.v11.i31.4857*
6. Ucmak H, Kokoglu OF, Celik M, et al. Intra-familial spread of hepatitis B virus infection in eastern Turkey. *Epidemiol Infect* 2007; 135:1338–1343. *doi: https://dx.doi.org/10.1017/S0950268807008011*
7. Mast EE, Margolis HS, Fiore AE, et al. A comprehensive immunization strategy to eliminate transmission of hepatitis B virus infection in the United States recommendations of the Advisory Committee on Immunization Practices (ACIP) part 1: immunization of infants, children, and adolescents. *MMWR Recomm Rep* 2005; 54:1-31. Available at: *https://www.ncbi.nlm.nih.gov/pubmed/16371945. No doi*
8. Petersen NJ, Barrett DH, Bond WW, et al. Hepatitis B surface antigen in saliva, impetiginous lesions, and the environment in two remote Alaskan villages. *Appl Environ Microbiol* 1976; 32:572-574. Available at: *https://www.ncbi.nlm.nih.gov/pubmed/791124 No doi*
9. Weinbaum CM, Williams I, Mast EE, Wang SA, et al. Recommendations for identification and public health management of persons with chronic hepatitis B virus infection. *MMWR Recomm Rep* 2008; 57:1-20. Available at: *https://www.ncbi.nlm.nih.gov/pubmed/18802412 No doi*

Case Report

Dengue haemorrhagic fever in late pregnancy causing maternal and intrauterine foetal death – A case report

SA Gunawardena[1], V Dias[1], O Amarajeewa[2], P Ellangage[3],
S Gunarathna[3], P Gunaratne[3], H Gunawardhana[4], S Jayasuriya[4]

Sri Lankan Journal of Infectious Diseases 2019 Vol.9 (1):88-94

DOI: http://dx.doi.org/ 10.4038/sljid.v9i1.8215

ABSTRACT

Dengue during pregnancy carries a higher risk of maternal and foetal complications, either through haemodynamic instability from disruption of the placental perfusion or through vertical transmission to the foetus.

A previously healthy 29-year-old primigravidae with a POA of 34 weeks presented with one day fever to a tertiary hospital. NS 1 antigen for dengue was positive. She entered the critical phase the following day and her platelet count dropped to 3000/ml by day 3. Intra uterine death was diagnosed on day 4. She continuously deteriorated and died on day 7. At autopsy, gross bleeding manifestations were noted in the mother, along with bilateral pleural effusions, massive sub endocardial haemorrhages and an enlarged liver with sub capsular haemorrhages. The placenta was devoid of any haemorrhages or infarcts. Sub-aponeurotic and subarachnoid haemorrhages and 50ml of blood within the thoracic and peritoneal cavities were found in a mature female foetus with minimal signs of maceration. Laboratory confirmation of foetal dengue virus infection was not possible.

This is a rare case where fatal haemorrhagic manifestations were seen in both the mother and the foetus suggesting vertical transmission. The autopsy findings highlight the unpredictable haemodynamic changes in the uterine circulation which severely hinder dengue management during pregnancy. Dengue infection, especially in late pregnancy, can lead to unpredictable fatal outcomes. The potential benefit of performing an emergency caesarean in such cases should be further explored.

[1]*Department of Forensic Medicine and Toxicology, Faculty of Medicine, University of Colombo.*
[2]*Faculty of Medicine, University of Peradeniya*
[3]*Faculty of Medicine, University of Sri Jayewardenepura*
[4]*Faculty of Medicine, University of Sri Jayewardenepura*

Address for correspondence: Dr. SA Gunawardena, Department of Forensic Medicine and Toxicology, Faculty of Medicine, University of Colombo, Tel: +94714958897 Email: sameera@fortox.cmb.ac.lk

http://orcid.org/0000-0001-9562-0533

Received 29 April 2018 and revised version accepted 6 February 2019

Keywords: Infectious diseases in pregnancy, Vertical transmission, Maternal mortality, Tropical diseases

Introduction

Over 100 million cases of dengue are reported worldwide each year, mainly from tropical countries.[1] By November 2017, 167,198 dengue cases including 215 deaths were reported in Sri Lanka.[2,3] Clinical symptoms ranged from asymptomatic fever or undifferentiated fever to more severe forms of Dengue Haemorrhagic Fever (DHF) or Dengue Shock Syndrome (DSS).[4] Dengue during pregnancy carries a higher risk of maternal and foetal complications, such as pre-term delivery, intra uterine death, miscarriages and prolonged bleeding during deliveries.[5] It is believed that dengue infection can disrupt the physiological changes in pregnancy causing haemodynamic instability in placental perfusion[6,7] or direct infection of the foetus through vertical transmission.[8]

Case presentation

A previously healthy 29-year-old primigravid female in her 35th week of an uneventful pregnancy presented with fever for one day to a tertiary care hospital. On admission, she had a temperature of 100.8 °F with arthralgia, myalgia and shortness of breath and a blood pressure of 130/80 mmHg. NS 1 dengue antigen test was positive. She entered the critical phase on day 2 and developed gum bleeding, haematuria and bilateral pleural effusions. Her platelet count dropped from 135,000/mL to 12,000/mL by day 3 to 3,000/mL by day 4, which coincided with settling of fever and a progressive rise in haematocrit and liver enzymes. The foetal heart sounds drastically dropped on day 4 and intra-uterine death was confirmed by ultra-sound scan. Her condition continued to deteriorate, and she died on day 7.

Autopsy revealed blood stained fluid in the right pleural and peritoneal cavities along with sub-mucosal haemorrhages in the larynx. The liver was enlarged, weighing 1300g, and had sub-capsular haemorrhages (Figure 1). There were extensive sub-endocardial haemorrhages in the left ventricle (Figure 2). The brain showed mild subdural haemorrhage along with cerebral oedema and congestion.

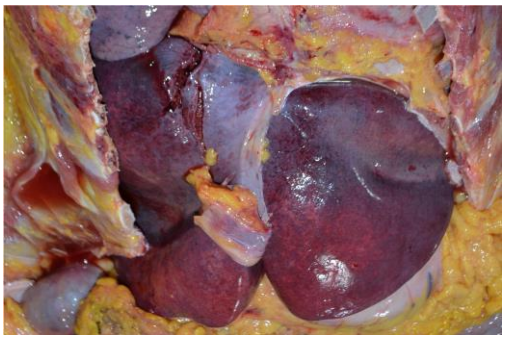


Figure 1: The liver of the deceased mother which was enlarged and bulging beyond the subcostal margin. Extensive subcapsular haemorrhages were seen throughout the parenchyma

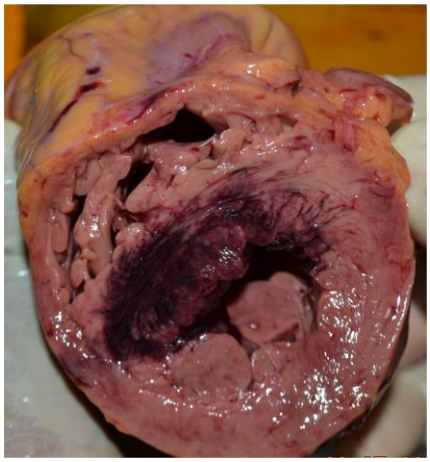


Figure 2: Massive subendocardial haemorrhage in the maternal heart

A female foetus compatible with 35 weeks of gestation was found inside the uterus. There were minimal signs of maceration. The umbilical cord was dark red and fleshy with no visible strictures or strangulations. The placenta did not show any haemorrhages or infarcts. Dissection of the foetus revealed subgaleal haemorrhage overlying the sagittal suture and a moderate amount of subarachnoid haemorrhage accumulated within the sulci (Figure 3). Both the thoracic and peritoneal cavities contained approximately 50ml of blood. There were no other visible haemorrhagic lesions. The macroscopic appearance and the distribution of the haemorrhages as well as the lack of other signs of maceration ruled out the possibility of post-mortem autolysis, hypostasis or congestion.

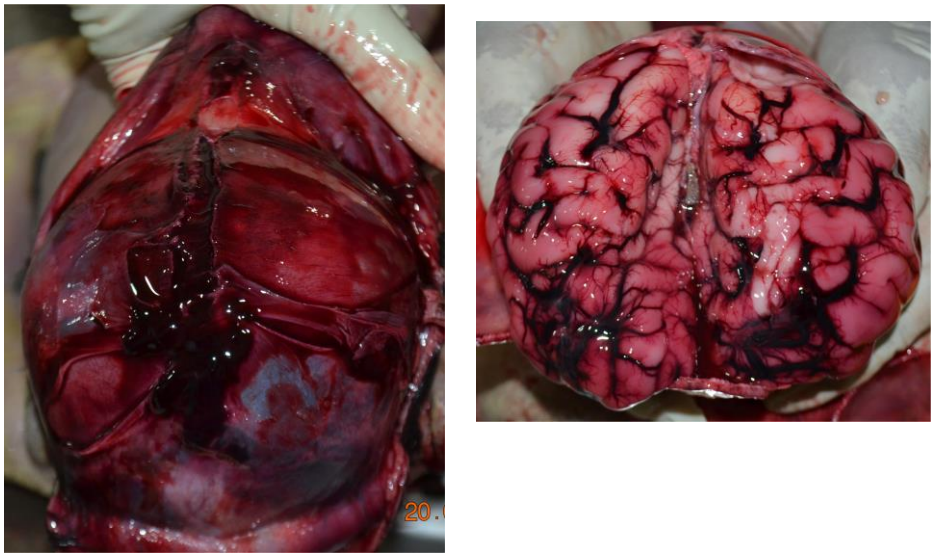


Figure 3: Subgaleal (left) and subarachnoid haemorrhage (right) were seen in the foetus

Due to practical difficulties and time constraints, the autopsy was limited to macroscopic examination. Serological and histopathological investigations were not carried out.

The cause of the maternal death was given as dengue haemorrhagic fever in pregnancy based on the antemortem clinical records and the macroscopic autopsy findings.

The timeline of the patient's clinical course is given in Figure 4.

29-year-old primigravid female in 35th week of pregnancy

Time	Clinical state		Platelets	Foetal heart sounds
Day 1	Admission with history of fever for 1 day	Fever, arthralgia, myalgia, dyspnoea, BP – 130/80 mmHg NS-1 positive	135000/mL	140-150 bpm
Day 2	Enters critical phase	Gum bleeding, haematuria pleural effusions		140-150 bpm
Day 3			12000/ml 55000/ml	~120 bpm
Day 4	Transferred to Infectious Diseases Hospital	Fever settles haematocrit & liver enzymes rise	3000/ml	Absent (IUD confirmed by ultrasound)
Day 5 & 6		Continuous deterioration		
Day 7	Death			

Figure 4: Timeline of clinical progression

Discussion

Dengue haemorrhagic fever (DHF) is one of the more life-threatening manifestations of dengue infection, which is believed to occur from immune mediated alterations in vascular permeability, thrombocytopenia and reduced fibrogen.[9] Pregnancy increases the risk of mortality from DHF[10], especially when the infection occurs in late pregnancy.[7,11] Vertical transmission of dengue infection from mother to foetus is a well-known phenomenon[12-16] and the reported incidence ranges from 1.6%[17] to 10.5%.[18] Waduge et al believe that the endothelial damage and increased vascular permeability in DHF leads to a disruption of the placental barrier, permitting entry of the dengue virus[6] as well as a multitude of parenchymal and interstitial changes in the placenta which lead to foetal hypoxia.[19-23]

None of the reported cases of miscarriages, pre-term deliveries and still births following dengue infection during pregnancy[5,6] refer to haemorrhagic manifestations in the foetus or new born with the exception of one case where the baby died from intracerebral haemorrhage 6 days after birth.[24] The development of haemorrhage within a foetus, particularly intracranial haemorrhage, is a phenomenon that is not clearly understood and has an estimated incidence of 1 in 10000 pregnancies.[25] Established causes are mostly acquired or inherited coagulopathies or maternal trauma.[26,27] However, there is strong evidence to suggest intrauterine hypoxia as a major cause.[28,29]

In this patient, the mother was in her 35th week of gestation with a viable healthy foetus when she was admitted with dengue fever. Her deterioration was extremely rapid with bleeding manifestations and intrauterine death occurring 3-4 days after the onset of symptoms. It is not clear

whether the haemorrhages detected in the foetus were a result of transplacental virus transmission triggering DHF in the foetus or due to hypoxic changes from placental insufficiency caused by haemodynamic changes within the uterine circulation. Unfortunately, the lack of serological and histopathological testing in this case restricts the possibility of deeper analysis.

This case report however clearly highlights the challenges in managing dengue during pregnancy. The pleural effusions and subendocardial haemorrhages found in the mother indicate a high degree of haemodynamic instability, possibly alternating between fluid overload and hypovolemia. Fluid management in a DHF patient is highly complicated, even without the added burden of sudden variations in placental and foetal circulation. It is perhaps worthwhile considering if this death could have been prevented if a caesarean section had been performed upon admission. This is an important consideration for future obstetric management in near-term pregnancies presenting with dengue infection, especially in tropical countries where dengue is rampant.

Conclusion

This case report presents a rare occurrence of an intrauterine and maternal death following DHF in pregnancy. This was despite the mother presenting herself to a tertiary hospital on the first day of fever and being provided the expected care. At autopsy, haemorrhagic manifestations were seen in the foetus which raises the possibility of vertical transmission of the virus, resulting in an intrauterine manifestation of fatal dengue haemorrhagic fever. Serological confirmation however was not available. Autopsy findings in the mother indicate the unpredictable nature of haemodynamic changes in the placental and foetal circulation which severely hindered successful management of her condition. Considering the high risk of maternal and foetal complications that are being reported, it is worthwhile considering if dengue fever in late pregnancies should be an indication for early termination before the onset of haemodynamic complications.

References

1. Moss PJ, Irving WL, Anderson J. Infectious diseases, tropical medicine and sexually transmitted diseases. In: Parveen K, Clarke M, editors. Kumar and Clarke's Clinical Medicine. 8th Ed. Saunders Elsevier. 2012. p. 73-195
2. Epidemiology Unit, Ministry of Health. Disease surveillance, trends. Distribution of notification(H399) Dengue Cases by Month. 2017. http://www.epid.gov.lk/web/index.php?option=com_casesanddeaths&Itemid=448&lang=en. Accessed on 08th Nov 2017.
3. World Health Organisation. Dengue Fever Sri Lanka. Emergencies, Preparedness, Response. 2017. http://www.who.int/csr/don/19-july-2017-dengue-sri-lanka/en/. Accessed on 08th Nov 2017
4. Malavige GN, Ogg GS. T cell responses in dengue viral infections. *Journal of Clinical Virology*. 2013; 58(4):605-11. *doi: https://doi.org/10.1097/01.inf.0000143644.95692.0e*
5. Basurko C, Carles G, Youssef M, Guindi WE. Maternal and foetal consequences of dengue fever during pregnancy. *European Journal of Obstetrics & Gynaecology and Reproductive Biology*. 2009; 147(1):29-32. *doi: https://doi.org/10.1016/j.ejogrb.2009.06.028*
6. Waduge R, Malavige GN, Pradeepan M, et al. Dengue infections during pregnancy: a case series from Sri Lanka and review of the literature. *Journal of Clinical Virology*. 2006; 37(1):27-33. *doi: https://doi.org/10.1016/j.jcv.2006.06.002*

7. Tsai HC, Lin CC, Hong NS, et al. Dengue virus infection in early gestation with delivery of an unaffected foetus and no vertical transmission. *Taiwanese Journal of Obstetrics and Gynaecology*. 2010; 49(1):112-4. *doi: https://doi.org/10.1016/S1028-4559(10)60023-X*
8. Sinhabahu VP, Sathananthan R, Malavige GN. Perinatal transmission of dengue: a case report. *BMC Research Notes*. 2014; 7(1):795. *doi: https://doi.org/10.1186/1756-0500-7-795*
9. Sellahewa KH. Pathogenesis of dengue haemorrhagic fever and its impact on case management. *ISRN Infectious Diseases*. 2013;2013:6 *doi:http://dx.doi.org/10.5402/2013/571646*
10. Machado CR, Machado ES, Rohloff RD, et al. Is pregnancy associated with severe dengue? A review of data from the Rio de Janeiro surveillance information system. *PLoS Neglected Tropical Diseases.* 2013; 7(5):e2217. *doi: https://doi.org/10.1371/journal.pntd.0002217*
11. Tan PC, Soe MZ, Lay KS, et al. Dengue infection and miscarriage: a prospective case control study. *PLoS Neglected Tropical Diseases*. 2012; 6(5):e1637. *doi: https://doi.org/10.1371/journal.pntd.0001637*
12. Chye JK, Lim CT, Ng KB, et al. Vertical transmission of dengue. *Clinical Infectious Diseases*. 1997; 25(6):1374-7. *doi:https://doi.org/10.1086/516126*
13. Thaithumyanon P, Thisyakorn U, Deerojnawong J, Innis BL. Dengue infection complicated by severe hemorrhage and vertical transmission in a parturient woman. *Clinical Infectious Diseases*. 1994; 18(2):248-9. *doi: https://doi.org/10.1093/clinids/18.2.248*
14. Fatimil LE, Mollah AH, Ahmed S, Rahman M. Vertical transmission of dengue: first case report from Bangladesh. *The Southeast Asian Journal of Tropical Medicine and Public Health*. 2003; 34(4):800-3. *PMID:15115091*
15. Perret C, Chanthavanich P, Pengsaa K et al. Dengue infection during pregnancy and transplacental antibody transfer in Thai mothers. *Journal of Infection*. 2005; 51(4):287-93. *doi: https://doi.org/10.1016/j.jinf.2004.10.003*
16. Sirinavin S, Nuntnarumit P, Supapannachart S, et al. Vertical dengue infection: case reports and review. *The Pediatric Infectious Disease Journal*. 2004; 23(11):1042-7. *doi: https://doi.org/10.1097/01.inf.0000143644.95692.0e*
17. Tan PC, Rajasingam G, Devi S, Omar SZ. Dengue infection in pregnancy: prevalence, vertical transmission, and pregnancy outcome. *Obstetrics & Gynecology*. 2008; 111(5):1111-7 *doi:https://doi.org/10.1097/AOG.0b013e31816a49fc*
18. Carles G, Talarmin A, Peneau C, Bertsch M. Dengue fever and pregnancy. A study of 38 cases in French Guiana [in French]. *Journal of Gynecology Obstetrics and Human Reproduction* (Paris) 2000; 29:758–62 *PMID:11139712*
19. Ribeiro CF, Lopes VG, Brasil P, et al. Perinatal transmission of dengue: a report of 7 cases. *The Journal of Pediatrics*. 2013; 163(5):1514-6. *doi: https://doi.org/10.1016/j.jpeds.2013.06.040*
20. Ribeiro CF, Silami VG, Brasil P, Nogueira RM. Sickle-cell erythrocytes in the placentas of dengue-infected women. *International Journal of Infectious Diseases*. 2012; 16(1):e72. *doi: https://doi.org/10.1016/j.jpeds.2013.06.040*
21. Miller J, Turan S, Baschat AA. Foetal growth restriction. *Seminars in Perinatology* 2008; 32(4): 274-280. *doi: https://doi.org/10.1053/j.semperi.2008.04.010*
22. Sharp AN, Heazell AE, Crocker IP, Mor G. Placental apoptosis in health and disease. *American Journal of Reproductive Immunology*. 2010; 64(3):159-69. *doi: https://doi.org/10.1111/j.1600-0897.2010.00837.x*
23. Heazell AE, Sharp AN, Baker PN, Crocker IP. Intra-uterine growth restriction is associated with increased apoptosis and altered expression of proteins in the p53 pathway in villous trophoblast. *Apoptosis*. 2011; 16(2):135-44. *doi: https://doi.org/10.1007/s10495-010-0551-3*
24. Chye JK, Lim CT, Ng KB, et al. Vertical transmission of dengue. *Clinical Infectious Diseases* 1997; 25:1374–7 *PMID:9431381*
25. Vergani P, Strobelt N, Locatelli A, et al. Clinical significance of foetal intracranial haemorrhage. *American Journal of Obstetrics and Gynecology*. 1996; 175(3 Pt 1):536-43.

doi: https://doi.org/10.1053/ob.1996.v175.a73598

26. Sherer DM, Anyaegbunam A, Onyeije C. Antepartum foetal intracranial haemorrhage, predisposing factors and prenatal sonography: a review. *American Journal of Perinatology*. 1998; 15(07):431-41. *doi: https://doi.org/10.1055/s-2007-993971*
27. Ghi T, Simonazzi G, Perolo A, et al . Outcome of antenatally diagnosed intracranial haemorrhage: case series and review of the literature. *Ultrasound in Obstetrics & Gynaecology*. 2003; 22(2):121-30. *doi: https://doi.org/10.1002/uog.191*
28. Achiron R, Pinchas OH, Reichman B, et al. Foetal intracranial haemorrhage: clinical significance of in utero ultrasonographic diagnosis. *British Journal of Obstetrics & Gynaecology*. 1993;100(11):995-9. *doi: https://doi.org/10.1111/j.1471-0528.1993.tb15140.x*
29. Geddes JF, Tasker RC, Hackshaw AK, et al. Dural haemorrhage in non-traumatic infant deaths: does it explain the bleeding in 'shaken baby syndrome'? *Neuropathology and Applied Neurobiology*. 2003; 29(1):14-22. *doi: https://doi.org/10.1046/j.1365-2990.2003.00434.x*

Case Report

Isolated facial nerve palsy: Rare manifestation of dengue haemorrhagic fever A case report

ARM Misthaq[1], S Pirasath[1], AGH Sugathapala[1]

Sri Lankan Journal of Infectious Diseases 2019 Vol.9 (1):95-97

DOI: http://dx.doi.org/10.4038/sljid.v9i1.8237

Abstract

Dengue is a common arboviral infection and is one of the tropical diseases which occur in Sri Lanka. Neurological manifestations due to dengue are very rare but can be caused by serotypes 2 and 3. Here we report of an isolated facial nerve palsy occurring as a manifestation of dengue haemorrhagic fever (DHF) in a young boy who presented with fever and constitutional symptoms. Haematological parameters were suggestive of dengue with dengue IgM being positive. Subsequently, he developed right side lower motor neuron type of facial nerve palsy. He was treated with a high dose of steroids and facial nerve stimulation therapy. He clinically recovered without residual weakness.

Keywords: Dengue fever, Facial nerve palsy, Lower motor neuron

Introduction

Dengue is a common arboviral infection and is among the tropical diseases which occur in Sri Lanka. Dengue virus has four serotypes. Neurological manifestations are very rare and are caused by serotypes 2 and 3.[1] Encephalopathy, meningitis, acute pure motor weakness, mononeuropathies, transverse myelitis, stroke, acute disseminated encephalomyelitis, Guillain-Barre Syndrome, hypokalemic paralysis and neuromyelitis optica are the recognized neurological manifestations associated with dengue fever.[2] There were few cases of isolated facial nerve palsy in dengue haemorrhagic fever which had been reported in literature in Sri Lanka.[3] Here we report of an isolated facial nerve palsy occurring as a manifestation of dengue haemorrhagic fever in a young boy.

Case report

A previously healthy 18 year old boy presented with fever, myalgia, arthralgia and headache of 3 days duration. He had no other systemic symptoms. On examination, he was febrile and flushed. His pulse rate was 92/minute and blood pressure 100/60mmHg without postural drop. He had no

[1]*Colombo South Teaching Hospital, Kalubowila, Sri Lanka.*

Address for correspondence: Dr. Selladurai Pirasath, Colombo South Teaching Hospital, Kalubowila, Sri Lanka

Telephone: +94775122995 Email: selladuraipirasath81@gmail.com *https://orcid.org/0000-0002-4274-4919*

Received 24 December 2018 and revised version accepted 17 March 2019

evidence of leakage on admission. His initial full blood count showed leukopenia (3250/mm^3) with predominant lymphocytes (36%) and thrombocytopenia (96,000/mm^3). His NS 1 antigen was positive on day 3 and dengue IgM antibody was positive on day 7. On the 6th day, his platelet count dropped to 56,000/mm^3 with a raised packed cell volume (42% to 48%). His white cell count dropped to 2840/mm^3 (neutrophils – 45%, lymphocytes – 42%). His liver enzymes were high (AST – 78 IU/l, ALT – 62 IU/l) but renal function tests were normal. His ultrasound showed moderate ascites and pleural effusion suggestive of the critical phase of dengue fever.

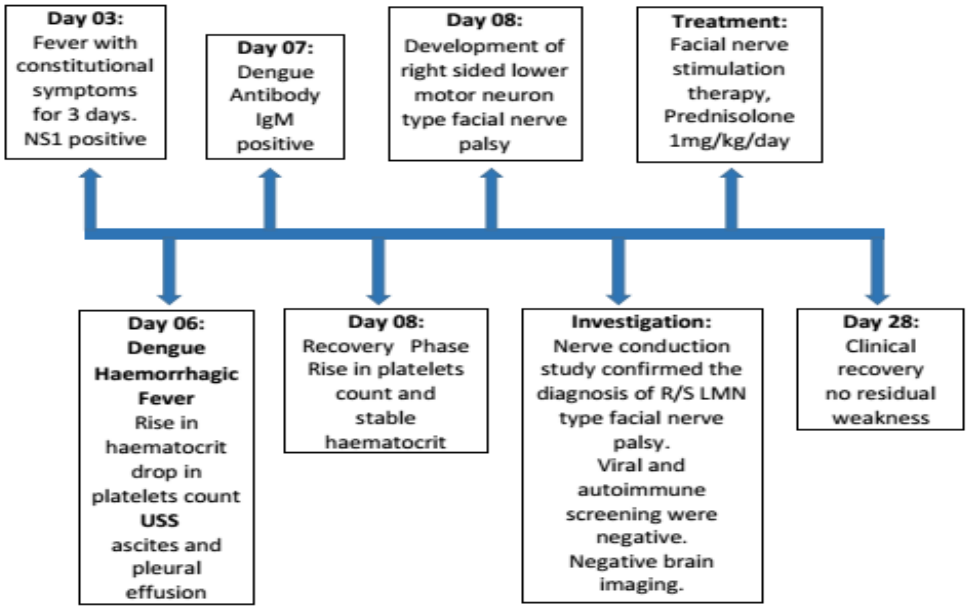
He was managed according to the national guidelines of dengue haemorrhagic fever published by the Ministry of Health, Sri Lanka.[4] He improved clinically, and platelet counts returned to normal limits.

On the 8th day, in recovery phase, he developed deviation of his mouth to the left side, difficulty in closing his right eye and wrinkling of the right side of his forehead. He had no limb weakness, facial numbness, swallowing difficulty, double vision, unsteady gait, earache or parotid swelling. On neurological examination, he had a right sided lower motor neuron type of facial nerve palsy. His nerve conduction study confirmed the diagnosis. Imaging of his brain was normal and inflammatory markers and autoimmune screening were negative. His work up for viral etiology (HSV, EBV and retroviral screening) was negative.

The patient was treated with prednisolone 1mg/kg/day for 10 days and facial nerve stimulation therapy. He was followed up at the clinic and he recovered without residual weakness at 4 weeks.

The timeline of the patient's clinical course is shown below.

Timeline of clinical progression



Discussion

Dengue is the most commonly encountered arboviral disease among tropical diseases in Sri Lanka. Neurological manifestations in dengue are a rare phenomenon but the incidence of unusual presentations and complications of this common viral infection is on the rise. Neurological manifestations are very rare and are usually seen with serotypes 2 and 3.[1] Neurological signs were first described in 1976 as atypical symptoms of dengue infection.[5] The pathogenesis of neurological complications and the contribution of viral and host factors are not well understood but can be related to neurotrophic effects of the virus, systemic effects of the infection or can even be immune mediated.[6]

The neurological complications of dengue virus infection are classified into three categories: metabolic disturbances (encephalopathy), viral invasion (encephalitis, meningitis, myositis, and myelitis) and autoimmune reactions (acute disseminated encephalomyelitis, neuromyelitis optica, optic neuritis, myelitis, encephalopathy, and Guillain-Barre syndrome).[2]

Dengue is gradually becoming a major public health problem worldwide. A growing number of related studies will increase awareness and understanding of the neurological complications of dengue infection. Physicians will continue to play important roles in its diagnosis and treatment. A high level of suspicion can lead to rightful early diagnosis and prompt timely management leading to a significant improvement in mortality and morbidity in cases of neurological complications due to dengue. It should be considered as a cause for lower motor neuron type facial nerve palsy in an endemic area in clinical practice.

Conflict of Interest: Authors declare no conflict of interest

Ethics: Informed and written consent for publication was obtained from the patient

References

1. Chowdhury RN, Siddiqui MR, Mahbub MS, et al. Dengue fever as a cause of acute disseminated encephalomyelitis (ADEM). *J Medicine* 2011; 12:185-187. *doi: https://doi.org/10.3329/jom.v12i2.8428*
2. Murthy JM. Neurological complication of dengue infection. *Neurol India* 2010; 58:581-4. *doi: https://doi.org/10.4103/0028-3886.68654*
3. Sunantha S, Peranantharajah T, Nisahan B. Isolated lower motor neuron type facial nerve palsy: An unusual manifestation of dengue haemorrhagic fever. *Jaffna Medical Journal* 2018; 30(1):35.6. *doi: http://doi.org/10.4038/jmj.v30i1.11*
4. Ministry of Health, Sri Lanka. National guidelines. Guidelines on management of dengue fever &. dengue haemorrhagic fever. *http://www.epid.gov.lk/web/images/pdf/Publication/guidelines_for_the_management_of_df_and_dhf_in_adults.pdf*
5. Sanguansermsri T, Poneprasert B, Phornphutkul B, et al. Acute encephalopathy associated with dengue infection. Bangkok: SEAMEO TROPMED; 1976. *No doi*
6. Domingues R. B., Kuster G. W. Diagnosis and management neurologic manifestations associated with acute dengue virus infection. *J. Neuroinfect. Dis* 2014; 5:138 *doi: https://doi: 10.4172/2314-7326.1000138.*