COMPARISON OF NEUTROPHIL EXTRACELLULAR TRAP INDUCTION AND REACTIVE OXYGEN SPECIES PRODUCTION BETWEEN NON-CAPSULATED AND CAPSULATED STRAINS OF *BURKHOLDERIA THAILANDENSIS*

Donporn Riyapa[1], Veerachat Muangsombut[2], Sujintana Janesomboon[2], Vanaporn Wuthiekanun[3], Joanne M Stevens[4] and Sunee Korbsrisate[2]

[1]Center for Research and Innovation, Faculty of Medical Technology, Mahidol University, Nakhon Pathom; [2]Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University; [3]Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; [4]Division of Infection and Immunity, The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Midlothian, United Kingdom

Abstract. Polymorphonuclear neutrophils (PMNs) play a role in host defense by eliminating infectious pathogens through phagocytic activity and degranulation. Neutrophil extracellular traps (NETs) are an additional antimicrobial mechanism of PMNs that involve releasing nuclear DNA and antimicrobial proteins to entrap and kill microbes. The generation of NETs requires reactive oxygen species (ROS) production *via* the activation of NADPH oxidase complex with certain NET-inducing stimuli. However, little is known about *Burkholderia thailandensis*-induced NETs. In this study, we aimed to compare NET formation and ROS levels between typical *B. thailandensis* strain E264 (non-capsulated strain) and a variant *B. thailandensis* strain E555 (capsulated strain). *B. thailandensis* strain E555 was included in this study due to its production of a capsule similar to the pathogenic bacteria *Burkholderia pseudomallei*. We performed this comparison to determine if the capsule would result in a difference in NET formation. The amounts of NETs were measured using a fluorometric double-stranded DNA quantification assay and the ROS levels were measured by flow cytometry. At a multiplicity of infection (MOI) of 10, *B. thailandensis* strain E555 induced significantly less (193.0 ng/ml) NET formation than *B. thailandensis* strain E264 (285.4 ng/ml, $p<0.05$). Significantly lower ROS levels were produced by *B. thailandensis* strain E555 (MFI value 37.6) than *B. thailandensis* strain E264 (MFI value 63.4, $p<0.05$). Our results showed both strains of *B. thailandensis* induced NET formation and ROS levels. However, strain E555 induced less NET formation and produced lower ROS levels than strain E264, suggesting the capsule enabled *B. thailandensis* strain E555 to evade the induction and killing activity of NETs. Further studies are needed to determine if these differences are due to the capsule or other factors.

Correspondence: Dr Sunee Korbsrisate, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Wanglang Road, Bangkok 10700, Thailand.
Tel: +66 (0) 2418 0569; Fax: +66 (0) 2418 1636. E-mail: sunee.kor@mahidol.edu

Keywords: *Burkholderia thailandensis* E264, *Burkholderia thailandensis* E555, neutrophil extracellular traps, NETs, capsule

INTRODUCTION

Polymorphonuclear neutrophils (PMNs) are one of the first lines of host defense against invading pathogens (Nauseef and Borregaard, 2014). They are rapidly recruited from the circulation to an infection site to engulf and degrade pathogens *via* phagocytic capacity and cytotoxic mechanisms (Kolaczkowska and Kubes, 2013; Nauseef and Borregaard, 2014). In 2004, a new mechanism for extracellular microbial killing was described as neutrophil extracellular trap (NET) formation (Brinkmann *et al*, 2004). NET function can be initiated through PMN activation with certain signals, such as the presence of microbes, interleukin-8 (IL-8), and phorbol 12-myristate 13-acetate (PMA), which trigger the membrane-bound NADPH oxidase enzyme complex to produce reactive oxygen species (ROS) inside PMNs (Fuchs *et al*, 2007; Parker *et al*, 2012). The high concentration of ROS causes a cytoplasmic membrane rupture and results in extrusion of a network of nuclear DNA and granule proteins into the extracellular space to entrap and occasionally kill microbes (Fuchs *et al*, 2007; Lu *et al*, 2012). However, many pathogens have evolved mechanisms to evade NET-mediated entrapment and killing (Buchanan *et al*, 2006; Urban *et al*, 2006). It has been reported that a capsule protects *Streptococcus pneumoniae* against the entrapment of NETs (Wartha *et al*, 2007).

Burkholderia pseudomallei is a gram-negative facultative intracellular pathogen and the causative agent of melioidosis (White, 2003). It is closely related to *Burkholderia thailandensis*, a non-pathogenic relative (Brett *et al*, 1998). However, there are reports of patients in Thailand and the United States becoming infected with *B. thailandensis* due to the contamination of open wounds in ditches or open water, although the infections have not been reported to cause mortality (Lertpatanasuwan *et al*, 1999; Glass *et al*, 2006). *B. thailandensis* is a gram-negative saprophytic rod found in soil and stagnant water in Southeast Asia, Northern Australia (Gee *et al*, 2008) and some other areas. *B. thailandensis* shares most virulence factors and genomic similarity with *B. pseudomallei* (Yu *et al*, 2006; Haraga *et al*, 2008). Hence, *B. thailandensis* has been proposed to be a useful surrogate for *B. pseudomallei* studies because it does not require a biosafety level 3 containment facility. One standard *B. thailandensis* strain utilized in laboratory research is E264. *B. thailandensis* strain E555 was isolated from environmental samples in Cambodia and Thailand (Sim *et al*, 2010; Hantrakun *et al*, 2018). This strain, encoding a *B. pseudomallei*-like capsule, has 96% similarity to the antigenic manno-heptose capsule of *B. pseudomallei* (Sim *et al*, 2010). The E555 strain also elicits protective immunity in *B. pseudomallei*-challenged mice (Sim *et al*, 2010; Scott *et al*, 2013), which may be due to expression of a similar immunogenic capsule. On genomic analysis, *B. thailandensis* strain E555 is more closely related to *B. pseudomallei* than *B. thailandensis* strain E264 (Sim *et al*, 2010).

B. pseudomallei can induce NET formation *via* NADPH oxidase activation and a capsule-deficient mutant of *B. pseudomallei* induces the elevated levels of extracellular DNA upon infection of PMNs (Riyapa *et al*,

2012). However, little is known about the interaction between *B. thailandensis* and human PMNs, particularly the presence of NET formation and ROS production among the different strains. The objective of the present study was to compare the ability of *B. thailandensis* strain E264 (non-capsulated strain) and *B. thailandensis* strain E555 (capsulated strain) to induce NET formation and intracellular ROS in human PMNs. The levels of NETs and ROS production by infected PMNs were determined by fluorometric double-stranded DNA quantification assay and flow cytometry, respectively.

MATERIALS AND METHODS

Studied bacterial strains

B. thailandensis strains E264 and E555 were kindly provided by the Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium ATCC 13311), which was previously reported to induce NET formation (Brinkmann *et al*, 2004) was included as a positive control for NET release in this study. The *Burkholderia* strains and *S.* Typhimurium were grown on Luria-Bertani (LB) agar or in broth (TM MEDIA, Delhi, India) at 37°C for 16 hours.

B. thailandensis preparation

The *B. thailandensis* was harvested after culturing for 16 hours and then washed twice with phosphate-buffered saline, pH 7.4 (PBS; Merck, Darmstadt, Germany). The number of bacteria was measured by spectrophotometry and counting. The bacterial concentration was adjusted to a multiplicity of infection (MOI) of 1 and 10.

To study the effect of *B. thailandensis* products on NET formation, bacterial supernatant was prepared by sterilizing the bacteria by syringe filtration through a 0.22-μm filter prior to mixing with purified PMNs (Pilsczek *et al*, 2010).

Polymorphonucleocyte isolation

The PMNs used for this study were obtained from human blood of subjects who gave written informed consent to participate in this study. This study was approved by the Mahidol University Central Institutional Review Board (MU-CIRB 2018/133.0507). The inclusion criteria for study subjects and methods of collecting the blood has been described previously (Chanchamroen *et al*, 2009). Human peripheral venous blood was collected into sterile Vacutainers (BD Biosciences, Franklin Lakes, NJ) containing sodium heparin as an anticoagulant. This whole blood was then mixed with 3.0% dextran T-500 (Pharmacosmos, Holbaek, Denmark) for 20 minutes at room temperature (RT) to sediment the erythrocytes. The leukocyte-enriched supernatant was collected and the layer of PMNs was then separated using Ficoll-Hypaque (Sigma, St. Louis, MO) density gradient centrifugation. Erythrocytes were removed using hypotonic lysis with sterile water. Isolated PMNs were then suspended in RPMI-1640 medium (Gibco BRL, Grand Island, NY) combined with 10% fetal bovine serum (FBS; Gibco BRL). The purity of the PMNs was evaluated using a differential count stained with Giemsa and with flow cytometry and found to be 95%. The viability of the PMNs was determined using trypan blue exclusion and found to be >98%.

Polymorphonucleocyte/studied bacteria culture preparation

The PMNs were then incubated at a concentration of 2.5×10^6 cells/ml with either *B. thailandensis* strain E264 or *B. thailandensis* strain E555 (MOIs of 1 and 10) or in bacterial culture supernatant for

90 minutes at 37°C in a 5% CO_2 incubator. The PMNs were also mixed with *S.* Typhimurium at MOIs of 1 and 10 along with phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO) at a concentration of 100 nM as a positive control for NET induction. Restriction enzymes *Eco*RI and *Hind*III (Thermo Scientific, Rockford, IL) at a concentration of 0.02 units/μl were added to the cultures for 2 hours at 37°C to digest the extracellular DNA released by stimulated PMNs. The enzymatic reaction was then stopped by adding EDTA (Merck, Darmstadt, Germany) to give a concentration of 5 mM and then incubated for 15 minutes at 65°C.

To quantify the amount of expelled DNA, the culture supernatants were collected and measured in duplicate for DNA concentration using the Picogreen dsDNA quantification kit (Molecular Probes, Eugene, OR) following with the manufacturer's instructions. The samples were detected at an excitation wavelength of 480 nm and an emission wavelength of 520 nm using spectrofluorometer (Infinite M200 PRO, Tecan, Germany).

Intracellular ROS assay

It was reported that intracellular ROS production is essential to induce NET formation (Fuchs *et al*, 2007). To determine the level of intracellular ROS, the PMNs (2.5×10^6 cells/ml) were incubated with either *B. thailandensis* strains E264 or E555 at MOIs of 1 and 10 for 90 minutes at 37°C. The PMNs were also mixed with *S.* Typhimurium at MOIs of 1 and 10 and PMA at a concentration of 100 nM as positive controls. After stimulation, 25 μl of a 2,800 ng/ml dihydroethidium (DHE) solution (Sigma, St. Louis, MO) was added into the cultures for 10 minutes at 37°C in the dark. The cultures were then washed twice with PBS and then fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) prior to analysis by flow cytometry (using a FACSCanto II; BD Bioscience, San Diego, CA). A change of mean fluorescence intensity (MFI) in DHE fluorescence reflects the intracellular accumulation of ROS levels.

Blocking of NADPH oxidase assay

To study signaling pathway of NET formation in *B. thailandensis* infection, the PMNs were pretreated with 10 μM diphenyleneiodonium chloride (DPI; Sigma, St. Louis, MO) to inhibit NADPH oxidase activity for 30 minutes at 37°C and *B. thailandensis* strains E264 and E555 at MOI of 10 were then added into the cultures for 90 minutes at 37°C. *S.* Typhimurium and PMA (100 nM) were included as controls. The cultures were then stained with DHE and analyzed by flow cytometry.

Statistical analysis

Statistical analysis was determined using a two-tailed, unpaired *t*-test by GraphPad Prism statistical program (GraphPad 5, San Diego, CA). All data were presented as means ± standard error of at least three independent experiments. Statistical significance was set at $p< 0.05$.

RESULTS

The means [± standard error of means (SEM)] in the quantification of the extracellular nuclear DNA from the PMNs infected by either *B. thailandensis* strain E264 or *B. thailandensis* strain E555 are shown in Fig 1A. The release of NETs was significantly higher (p=0.0034) in *B. thailandensis* strain E264 (156.4 ng/ml) than *B. thailandensis* strain E555 infection (107.8 ng/ml) at an MOI of 1. For *B. thailandensis* infection at an MOI of 10, *B. thailandensis* strain E264 (285.4 ng/ml) also induced significantly higher (p=0.0363) levels of NET formation than strain E555 (193.0

ng/ml). There was a significant increase of NET formation in *S.* Typhimurium and *B. thailandensis* strain E264 at an MOI of 10 (p=0.0061 and 0.0091, respectively) when compared to PMA stimulation as a positive control for NET formation.

The means ± SEM for studying the effect of *B. thailandensis* products on NET formation are shown in Fig 1B. The bacterial supernatants from *S.* Typhimurium, *B. thailandensis* strain E264, and strain E555 significantly induced (p=0.0060, 0.0041 and 0.0127, respectively) the releases of NETs when compared to medium control. However, there was no significant difference (p=0.6165) in NET formation between *B. thailandensis* strain E264 and *B. thailandensis* strain E555.

Representative histogram plots of ROS levels in *B. thailandensis*-infected PMNs (grey region) compared to uninfected PMNs (black line) are shown in Fig 2A. The decreased ROS levels in the PMNs infected by *B. thailandensis* strain E555 were observed when compared to *B. thailandensis* strain E264-infected PMNs. The comparisons of the relative mean fluorescence intensities (MFI) of ROS levels in the PMNs from four individual subjects infected by *B. thailandensis* strain E264 and *B. thailandensis* strain E555 are shown in Fig 2B. At an MOI of 1, there was a significant increase of ROS levels (p=0.0131) in *B. thailandensis* strain E264-infected PMNs (MFI value 33.6) when compared to the PMNs infected by strain E555 (MFI value 20.7). At an MOI of 10, *B. thailandensis* strain E264 (MFI value 63.4) also induced significantly higher levels of ROS (p=0.0031) than *B. thailandensis* strain E555 (MFI value 37.6). For positive control, there was a significant increase of ROS levels (p<0.05) in all bacteria at an MOI of 1 and *B. thailandensis* strain E555 at an MOI of 10 when compared to PMA-stimulated PMNs.

After PMN pretreatment with NADPH oxidase inhibitor DPI, the productions of ROS in the PMNs infected by *B. thai-*

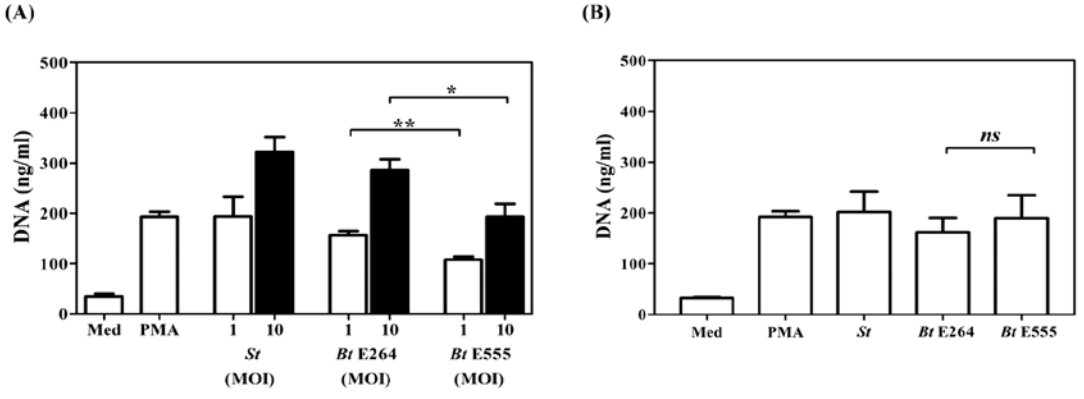

Fig 1-Comparison of NET levels in *B. thailandensis* infections. A). The levels of NETs induced by *S.* Typhimurium (*St*), *B. thailandensis* strains E264 (*Bt* E264), and strain E555 (*Bt* E555) infections at MOIs of 1 (white bar) and 10 (black bar) compared to medium control (Med) and PMA stimulation. B). The levels of NETs induced by bacterial products. Bar is mean ± standard error of mean (SEM) of four individual subjects. Significant differences by unpaired *t*-test. *p<0.05, **p<0.01, and NS (not significant).

landensis strains E264 and E555 were significantly inhibited (p=0.0008 and 0.0110, respectively) as shown in Fig 3A. For NET formation, the reductions of the means and standard error of means in DPI-pretreated PMNs are shown in Fig 3B. The DPI-pretreated PMNs significantly reduced the levels of NETs in *B. thailandensis* strains E264 and E555 (p=0.0001 and 0.0008, respectively). However, there was

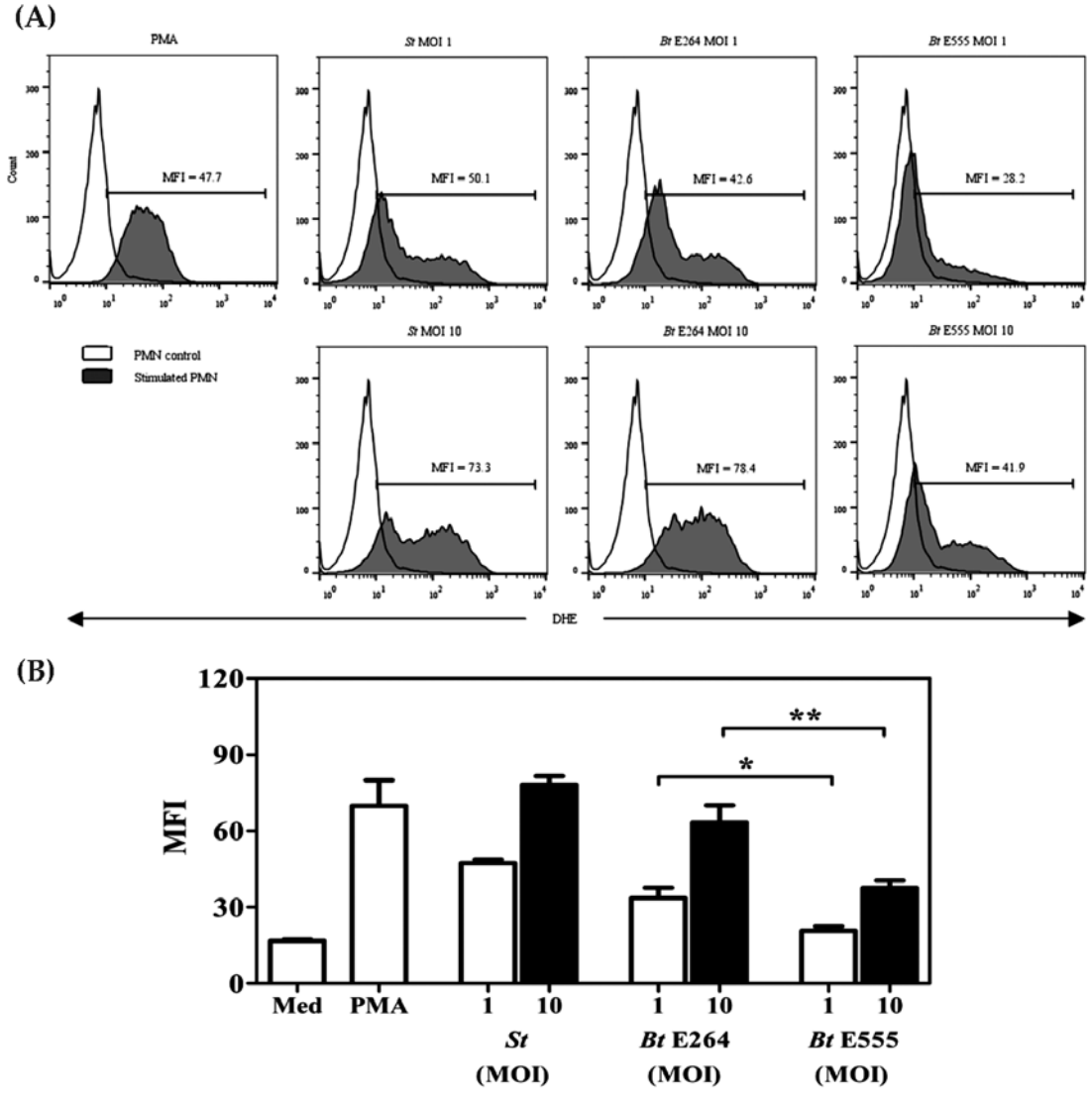

Fig 2-Comparison of ROS levels in *B. thailandensis* infections. A). Representative histograms showed the ROS levels in the PMNs infected *S.* Typhimurium (*St*), *B. thailandensis* strains E264 (*Bt* E264), and strain E555 (*Bt* E555) infections at MOIs of 1 and 10 compared to medium control (Med) and PMA stimulation. B). The relative levels of ROS obtained from four individual subjects are presented as the mean fluorescence intensities (MFI). Bar is mean ± standard error of mean (SEM). Significant differences by unpaired *t*-test. *p<0.05 and **p<0.01.

no significant difference (p=0.6165) in NET formation between *B. thailandensis* strain E264 and *B. thailandensis* strain E555. After PMA stimulation as a positive control, the DPI-pretreated PMNs significantly reduced the ROS levels and NET formation (p=0.0017 and 0.0012, respectively) when compared to the unpretreated PMNs.

DISCUSSION

In this present study, we found that *B. thailandensis* strain E555 (capsulated strain) induced significantly lower NET levels than *B. thailandensis* strain E264 (non-capsulated strain), suggesting the capsule of *B. thailandensis* strain E555 could have evaded PMN function by reducing the formation of NETs. Since the major difference between these two strains is the capsule, this may be the reason for the difference. A previous study demonstrated that the expression of capsule in *B. thailandensis* strain E555 is closely related to *B. pseudomallei* (Sim *et al*, 2010). Most strains of *B. thailandensis* do not produce capsule but *B. thailandensis* strain E555 produces the capsule that can cross-react with an anti-*B. pseudomallei* capsule monoclonal antibody (Sim *et al*, 2010; Scott *et al*, 2013). This agrees with a previous study that found a *B. pseudomallei* wild-type strain containing the capsule induced a low level of NETs. However, a *B. pseudomallei* mutant strain lacking the capsule has been shown the elevation of NETs compared to the wild-type strain (Riyapa *et al*, 2012). The capsular component of *Cryptococcus neoformans* has been reported to contribute to reduction in NET formation (Rocha *et al*, 2015), suggesting the role of the capsule for inhibition of NET formation.

We also found a higher level NET formation in PMNs stimulated with *B. thailandensis* strain E264 (non-capsule) resulting in a significant increase in ROS, suggesting that NETs are dependent on ROS production in *B. thailandensis* infection. ROS induction in PMNs can result from activation by several signaling pathways (Kolaczkowska and Kubes, 2013).

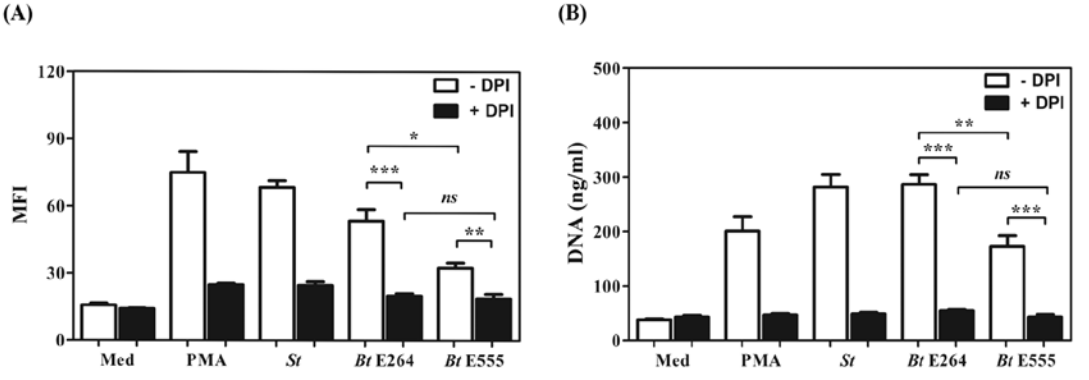

Fig 3-Inhibition of ROS and NET levels using the NADPH oxidase inhibitor. The PMNs were infected by *S.* Typhimurium (*St*), *B. thailandensis* strains E264 (*Bt* E264), and strain E555 (*Bt* E555) infections at an MOI of 10 in the absence (white bar) or presence (black bar) of NADPH oxidase inhibitor DPI. A). The levels of ROS production. B). The levels of NET formation. Bar is mean ± standard error of mean (SEM) of four individual subjects. Significant differences by unpaired *t*-test. *p<0.05, **p<0.01, ***p<0.001, and NS (not significant).

In our study, we found the ROS elevation generated *via* the NAPDH oxidase pathway. Blocking NAPDH oxidase by DPI reduced ROS and NET levels after *B. thailandensis* infections. A study of *B. pseudomallei* and *Staphylococcus aureus* found NET induction by these bacteria were also dependent on the NADPH oxidase pathway (Fuchs *et al*, 2007; Riyapa *et al*, 2012).

In summary, *B. thailandensis* strain E555 (capsule strain) inhibited the production of ROS-dependent NET formation by human PMNs. We conclude that the lower NET levels may be due to the capsule.

ACKNOWLEDGEMENTS

This study was supported by a Siriraj Grant for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University. Vanaporn Wuthiekanun was funded by the Wellcome Trust (Grant Number 089275/Z/09/Z. M.M). Sujintana Janesomboon was supported by a Siriraj Graduate Scholarship, Faculty of Medicine Siriraj Hospital, Mahidol University.

REFERENCES

Brett PJ, DeShazer D, Woods DE. *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. *Int J Syst Bacteriol* 1998; 48: 317-20.

Brinkmann V, Reichard U, Goosmann C, *et al.* Neutrophil extracellular traps kill bacteria. *Science* 2004; 303: 1532-5.

Buchanan JT, Simpson AJ, Aziz RK, *et al.* DNase expression allows the pathogen group A *Streptococcus* to escape killing in neutrophil extracellular traps. *Curr Biol* 2006; 16: 396-400.

Chanchamroen S, Kewcharoenwong C, Susaengrat W, *et al.* Human polymorphonuclear neutrophil responses to *Burkholderia pseudomallei* in healthy and diabetic subjects. *Infect Immun* 2009; 77: 456-63.

Fuchs TA, Abed U, Goosmann C, *et al.* Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* 2007; 176: 231-41.

Gee JE, Glass MB, Novak RT, *et al.* Recovery of a *Burkholderia thailandensis*-like isolate from an Australian water source. *BMC Microbiol* 2008; 8: 54.

Glass MB, Steigerwalt AG, Jordan JG, Wilkins PP, Gee JE. *Burkholderia oklahomensis* sp. nov., a *Burkholderia pseudomallei*-like species formerly known as the Oklahoma strain of *Pseudomonas pseudomallei*. *Int J Syst Evol Microbiol* 2006; 56: 2171-6.

Hantrakun V, Thaipadungpanit J, Rongkard P, *et al.* Presence of *B. thailandensis* and *B. thailandensis* expressing *B. pseudomallei*-like capsular polysaccharide in Thailand, and their associations with serological response to *B. pseudomallei*. *PLOS Negl Trop Dis* 2018; 12: e0006193.

Haraga A, West TE, Brittnacher MJ, Skerrett SJ, Miller SI. *Burkholderia thailandensis* as a model system for the study of the virulence-associated type III secretion system of *Burkholderia pseudomallei*. *Infect Immun* 2008; 76: 5402-11.

Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* 2013; 13: 159-75.

Lertpatanasuwan N, Sermsri K, Petkaseam A, Trakulsomboon S, Thamlikitkul V, Suputtamongkol Y. Arabinose-positive *Burkholderia pseudomallei* infection in humans: case report. *Clin Infect Dis* 1999; 28: 927-8.

Lu T, Kobayashi SD, Quinn MT, Deleo FR. A NET outcome. *Front Immunol* 2012; 3: 365.

Nauseef WM, Borregaard N. Neutrophils at work. *Nat Immunol* 2014; 15: 602-11.

Parker H, Dragunow M, Hampton MB, Kettle AJ, Winterbourn CC. Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. *J Leukoc Biol* 2012; 92: 841-9.

Pilsczek FH, Salina D, Poon KK, *et al.* A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J Immunol* 2010; 185: 7413-25.

Riyapa D, Buddhisa S, Korbsrisate S, *et al.* Neutrophil extracellular traps exhibit antibacterial activity against *Burkholderia pseudomallei* and are influenced by bacterial and host factors. *Infect Immun* 2012; 80: 3921-9.

Rocha JD, Nascimento MT, Decote-Ricardo D, *et al.* Capsular polysaccharides from *Cryptococcus neoformans* modulate production of neutrophil extracellular traps (NETs) by human neutrophils. *Sci Rep* 2015; 5: 8008.

Scott AE, Laws TR, D'Elia RV, *et al.* Protection against experimental melioidosis following immunization with live *Burkholderia thailandensis* expressing a manno-heptose capsule. *Clin Vaccine Immunol* 2013; 20: 1041-7.

Sim BM, Chantratita N, Ooi WF, *et al.* Genomic acquisition of a capsular polysaccharide virulence cluster by non-pathogenic *Burkholderia* isolates. *Genome Biol* 2010; 11: R89.

Urban CF, Lourido S, Zychlinsky A. How do microbes evade neutrophil killing? *Cell Microbiol* 2006; 8: 1687-96.

Wartha F, Beiter K, Albiger B, *et al.* Capsule and D-alanylated lipoteichoic acids protect *Streptococcus pneumoniae* against neutrophil extracellular traps. *Cell Microbiol* 2007; 9: 1162-71.

White NJ. Melioidosis. *Lancet* 2003; 361: 1715-22.

Yu Y, Kim HS, Chua HH, *et al.* Genomic patterns of pathogen evolution revealed by comparison of *Burkholderia pseudomallei*, the causative agent of melioidosis, to avirulent *Burkholderia thailandensis*. *BMC Microbiol* 2006; 6: 46.