INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
08–343/ZIP/2010/12–5–777–780
http://www.fspublishers.org

Full Length Article

Disc Diffusion Based *In Vitro* Antibiotic Susceptibility of Recent Isolates of *Burkholderia mallei*

M. SAQIB[1], G. MUHAMMAD[1], A. NAUREEN†, A. SHAKOOR† AND SAJJAD-UR-REHMAN‡
Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan
†*Department of Clinical Medicine and Surgery, University of Veterinary and Animal Sciences, Lahore, Pakistan*
‡*Department of Microbiology, University of Agriculture, Faisalabad, Pakistan*
[1]Corresponding author's emails: profdrgm_pk@yahoo.com; drsaqib_vet@hotmail.com

ABSTRACT

In vitro susceptibilities of 43 non-archived recent isolates of Burkholderia mallei to 12 antimicrobials were determined by disc diffusion technique. All isolates were sensitive to chloramphenicol, co-trimoxazole, doxycycline and gentamicin but resistant to ampicillin and cephradine. The order of present resistance to amoxicillin, norfloxacin, oxytetracycline, ciprofloxacin, co-amoxiclav and enrofloxacin was 90.6 (n = 39), 20.9 (n = 9), 6.9 (n = 3), 6.9 (n = 3), 4.6 (n = 2) and 4.6 (n = 2), respectively. In the light of present results, chloramphenicol, co-trimoxazole, doxycycline, co-amoxiclav and enrofloxacin seem good candidate antibiotics for future planning of experimental therapeutic trials in target species (equids). Furthermore, this information can be useful in contingency disease managemental plans for incidences of deliberate release of *B. mallei* as terrorist attacks and in situation of laboratory associated *B. mallei* infections in humans.

Key Words: *Burkholderia mallei*; Glanders; Bio-warfare; Antibiotic susceptibility; Antibiotic resistance

INTRODUCTION

Burkholderia mallei is the etiologic agent of glanders, a disease primarily of equids, which is also communicable to man with fatal consequences. Glanders has been eradicated from most parts of the world. However, the disease still occurs in Asia, Africa and South America (OIE, 2008). During the last decade, the disease outbreaks were reported in Brazil, Eretria, Ethiopia, Iran, Iraq, India, Pakistan and United Arab Emirates (Dvorak & Spickler, 2008; Naureen *et al*., 2007). *Burkholderia mallei* is an established bioweapon that was used in different wars during the 20th century (Rotz *et al*., 2002). Since *B. mallei* is an agent of biological warfare/bio terrorism and since glanders is a reemerging disease, researchers are focusing on effective treatment protocols both in man and animals species and post exposure prophylaxis (Lopez *et al*., 2003).

Treatment of bacterial diseases is ideally guided by the results of *in vitro* antibiotic susceptibility. Much of the published work on *in vitro* susceptibility of *B. mallei* was carried out on 8 to 4 decades old archived isolates dating back mostly to pre-antibiotic era. Often times, these isolates might have been passaged in the laboratory creating prospects of loss of plasmids encoding for resistance to antibiotics (Muhammad *et al*., 1998a). During the last 20 years, only 2 reports on susceptibility of non-archived (recent) isolates of *B. mallei* that had been sub-cultured only a few times in laboratory were added to scientific literature (Al-Ani *et al*., 1998; Muhammad *et al*., 1998b).

As we are moving forward with our experimental therapeutic treatment trials in the natural host, data of antibiogram of recent *B. mallei* isolates is obviously needed. This manuscript documents the antibiotic susceptibility of recent isolates of *B. mallei* determined by disc diffusion method.

MATERIALS AND METHODS

***Burkholderia mallei* isolates:** Forty three isolates of *B. mallei* were recovered from glandered equines (horses, n = 67; mules, n = 16; & donkeys, n = 19) presented to Veterinary Teaching Hospital, Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan. Culture and identification of *B. mallei* was carried out following procedures described by Muhammad *et al*. (1998b). Briefly, nasal swab samples, mature skin nodule aspirates and venous blood samples from clinically diseased equids were cultured on brain heart infusion agar (BHI agar, Oxoid, UK) supplemented with sheep blood. Nasal swab samples were incubated for 3 h at room temperature in saline containing 3000 units of benzyl penicillin per mL before plating. When *B. mallei* originated from more than one type of samples from the same animal (nasal swabs, pus & blood), it was considered as one isolate. The representative colonies were screened for catalase, indole and colistin resistance. Irregularly stained Gram

To cite this paper: Saqib, M., G. Muhammad, A. Naureen, A. Shakoor and S.U. Rehman, 2010. Disc diffusion based *in vitro* antibiotic susceptibility of recent isolates of *Burkholderia mallei*. *Int. J. Agric. Biol.,* 12: 777–780

negative rods, indole negative and resistant to colistin were presumptively identified as those of *B. mallei*. Non-motile, triple sugar iron negative, arginine and gelatin positive isolates were finally confirmed as *B. mallei*. These isolates were further confirmed in polymerase chain reaction by targeting *Burkholderia* intracellular motility A gene (Ulrich *et al*., 2006).

Antibiotic susceptibility testing: *In vitro* antimicrobial susceptibility to 12 antibiotics/antimicrobials (Table I) was determined by disc diffusion method according to the standards described by British Society of Antimicrobial Chemotherapy (Andrew, 2001). Sensitivity agar plates were seeded with 100 µL of 10^6 colony forming unit per milliliter of test isolates, which was adjusted spectrophotometrically. The plates were incubated at 37±1°C for 30 h. The zones of inhibition were measured and compared with break points for *B. pseudomallei* and non-Enterobacteriaceae *Staphylococcus aureus* ATCC 25923, *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 isolates were used as quality control for antibiotics.

RESULTS AND DISCUSSION

Availability of antibiotic susceptibility data of *B. mallei* is an essential tool for antibiotic treatment and prophylaxis in the face of deliberate release of this bio-warfare agent. The results of *in vitro* sensitivity of *B. mallei* isolates are given in the Table I. The inhibitory zones of antibiotics against quality control isolates were within recommended ranges (Andrew, 2001). In the present study, all *B. mallei* isolates were resistant to ampicillin and cephradine, whereas 90.6 (n = 39), 6.9 (n = 3), 20.9 (n = 9), 4.6 (n = 2) and 6.9 (n = 3) percent isolates showed resistance to amoxicillin, oxytetracycline, norfloxacin, enrofloxacin and ciprofloxacin, respectively. Natural resistance of *B. mallei* to penicillin is well known since late 1960s (Mishankin & Goldberg, 1969). The resistance associated with alteration in bacterial penicillin binding proteins (PBP) has been reported in *Burkholderia* (Burns, 2006). Beta-lactamase gene (*Pen*A), which powers the resistance against penicillins and cephalosporins has also been identified in *B. mallei* ATCC 23344 strain (Tribuddharat *et al*., 2003). Resistance to ampicillin, cephradine and amoxicillin as observed in the present study might either be due to modification in PBP or the occurrence of *Pen*A (β lactamase) gene. The later assumption is further substantiated when amoxicillin with β-lactamase inhibitor (clavulanate) was tested against amoxicillin resistant isolates. This combination remarkably restored the activity of amoxicillin against resistant isolates. However, 4.6% isolates were still resistant (Table I). Our strain-record indicated that only few isolates demonstrated β-lactamase activity (unpublished data) possibly due to the use of less sensitive method of determination of β-lactamase activity (nitrocephin touch stick) and/or slow growing nature of the organism. In general, these results are in close agreement with those of previous studies (Al-Izzi & Al-Bassam, 1989; Antonov *et al*., 1991; Kenny *et al*., 1999; Thibault *et al*., 2004), wherein authors reported high resistance (MIC_{90}>64 µg/mL) in *B. mallei* to ampicilin, amoxicilin and to a few cephalosporins (cefazolin, cefsulodin, cefoxitin; MIC_{90}>64 - > 128). Contrary to our findings, American (Heine *et al*., 2001), British (Kenny *et al*., 1999) and French (Thibault *et al*., 2004) *Burkholderia* research groups reported no resistance to co-amoxiclav. However, one intermediately sensitive strain (MIC 16 µg/mL) has been observed (Thibault *et al*., 2004). Resistance against co-amoxiclav encountered in the present study may be attributed to a modification in chromosomally encoded β-lactamase (LiPuma, 2007).

Among tetercyclines, oxytetracycline showed good anti *B. mallei* activity although 6.9% (n = 3) isolates were found resistant to this antibiotic. No resistance was recorded against doxycycline. Similar results have also been reported previously (Al-Izzi & Al-Bassam, 1989; Manzeniuk *et al*., 1995; Heine *et al*., 2001; Thibault *et al*., 2004). The use of oxytetracycline reportedly delayed the Strauss reaction (orchitis), while chlorteteracycline was the most promising antibiotic among teteracylines in the treatment of experimental glanders (Nemato *et al*., 1961). About 5 decades ago, 3 cases of human glanders were effectively treated with oxytetracycline and chlortetracycline (Tezok, 1958). In a recent study, animal passage and re-isolation of *B. mallei* markedly altered MIC of doxycycline, that raises the concern that eventually true resistance could emerge against new tetracyclines (Heine *et al*., 2001).

Consistent with the previous findings (Batmanov, 1991), where 13 *B. mallei* isolates were highly resistant to norfloxacin but displayed susceptibility to ofloxacin and ciprofloxacin, a high proportion of resistance (20.9%) against norfloxacin noted in the present study discourages the use of this antibacterial in prospective treatment trials. Norfloxacin has also been found ineffective in experimental glanders therapeutic trial in guinea pig and hamster (Batmanov, 1991). Previously, Muhammad *et al*. (1998b) reported absolute sensitivity of *B. mallei* isolates (n= 13) to norfloxacin. These results might be due to the use of different concentration of norfloxacin and low volume of bacterial suspension used (adjusted parallel to 0.5 McFarland turbidity standards). Furthermore, mutation in *gyr*A gene could be another inciting factor (Burns, 2006) in isolates of present study. Iraqi workers (Al-Ani *et al*., 1998) reported susceptibility of 14 isolates to enrofloxacin. In their study interpretive criterion seems to be based on larger zone of inhibition of enrofloxacin rather than susceptibility breakpoint. In the light of results of the present study, it appears that enrofloxacin and ciprofloxacin may be useful candidate antibiotics for the therapy of glanders as both antimicrobials reportedly accumulate in phagocytic cells (where *B. mallei* resides), achieving intracellular concentrations higher than the extracellular levels (Carlier *et al*., 1990; Schoevers *et al*., 1999). In *B. mallei*, mutation

Table I: Antibiogram Profile and Comparative inhibition zone* of different antimicrobials against 43 isolates of *Burkholderia mallei* recovered from glanderous equids

Antimicrobials	Disk potency (µg)	Sensitivity break point (mm)	Diameter of vone of inhibition (mm)																		Resistance (%)
			<8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	
Ampicillin	25	≥ 18	24	-	17	2															100
Amoxicillin	25	≥ 18	9	20	6	1	3		4												90.6
Co-amoxiclav	30	≥ 18					2	-	2	24	3	1	3	5	1	2					4.6
Cephradine	30	≥ 12	42	1																	100
Chloramphenicol	30	≥ 21								5	3	9	17	1	3	1	-	1	1		0
Gentamicin	25	≥ 15						3	-	4	2	23	2	-	5	2	1	1			0
Oxytetracycline	30	≥ 19				1	1	1	1	-	13	20	-	-	-	3	3				6.9
Doxycycline	30	≥ 16							6	-	9	11	4	3	7	1	2				0
Co-trimoxazole	25	≥ 16						1	8	15	1	1	3	9	2	2					0
Norfloxacin	10	≥ 17			3	1	5	-	11	7	9	6	-	1							20.9
Enrofloxacin	5	≥ 18				2		6	4	17	2	9	-	1	1	1					4.6
Ciprofloxacin	5	≥ 21			2				1	5	3	21	2	7	1	-	1				6.9

*No of susceptible isolates are given against black background

derived resistance against fluroquinolones including oxolinic acid, norfloxacin, enoxacin, ofloxacin and ciprofloxacin has been reported (Stepanshin, 1994). Therefore, a careful rational selection of antibiotics will be needed in any experimental therapeutic trial against glanders.

In line with the results of the earlier studies (Al-Izzi & Al-Bassam, 1989; Antonov *et al*., 1991; Kenny *et al*., 1999; Heine *et al*., 2001; Thibault *et al*., 2004), trimethoprim-sulphamethoxazole (co-trimoxazole), gentamicin and chloramphenicol showed excellent *in vitro* activity against all isolates of *B. mallei.* Lozovaia (1989) reported good *in vitro* anti- *B. mallei* activity of sulphonamides (sulphamonomethoxine, sulphasalazine, sulphanilamide & sulphamethoxazole) in combination with trimethoprim (TMP). Treatment with sulphadiazine alone or combinations of sulfas with TMP was reportedly successful in human and laboratory animals, respectively (Howe & Miller, 1947; Ansabi & Minou, 1951; Batmanov, 1993).

Whole genome analysis of *B. pseudomallei* strain K96243 and *B. mallei* strain ATCC 23344 exposed a close relationship between these organisms and *B. mallei* is a clonal derivative of *B. psudomallei* as evidenced by multilocus sequence typing (Godoy *et al*., 2003; Harland *et al*., 2007). A multidrug efflux system-Amr-OprA-identified in *B. pseudomallei* (Moore *et al*., 1999) is responsible for high level resistance to aminoglycosides (gentamicin for one). Re-annotation and comparison of ATP-binding cassette systems of both organisms revealed that *B. mallei* lacked an Amr-OprA system, which most likely contributes to its sensitivity to aminoglycosides (Harland *et al*., 2007). Therefore, sensitivity of *B. mallei* to gentamicin is an inherent characteristic and this antibiotic can be considered in future experimental treatment plans for glanders. However, it is worth noting that *B. mallei* is an intracellular pathogen and use of this antibiotic may not give bacteriological cure as it would not attain desired therapeutic levels within the scavenger (macrophages/dendritic) cells.

Results of susceptibility of *B. mallei* to chloramphenicol have been inconsistent. Some researchers reported sensitivity (Heine *et al*., 2001; Thibault *et al*., 2004), while others documented resistance to this antibiotic (Al-Izzi & Al-Bassam, 1989; Kenny *et al*., 1999). Use of different formats of susceptibility and isolates might be the cause of this disparity. Chloramphenicol is well known for its bone marrow suppressive activity (Papich & Riviere, 2001) and use of this drug in glanders seems potentially dangerous, because *B. mallei* depletes bone marrow reserves in affected animals. Therefore, florfenicol, a fluorinated chloramphenicol derivative lacking undesirable effects needs to be investigated in future studies.

Antibiotic susceptibility by direct diffusion method (DDM) often differs from results obtained by E-test, agar and broth dilution technique, which give minimum inhibitory concentration (MIC). MIC statistics generates quantitative information rather than qualitative. DDM is used as a guideline for dosage determination as well as for antibiotic selection (Burrow *et al*., 1993). Discrepancies have been noted between results obtained by disc diffusion and E-test for *B. pseudomallei* (Wuthiekanun *et al*., 2005). To know more precisely about susceptibility data (MIC) and selection of antibiotic for experimental glanders treatment, the isolates of present study need to be tested by other susceptibility formats (agar dilution for one which is considered a gold standard for susceptibility testing).

REFERENCES

Al-Ani, F.K., O.F. Al-Rawashdeh, A.H. Ali and F.K. Hassan, 1998. Glanders in horses: clinical, biochemical and serological studies in Iraq. *Vet. Archiv*., 68: 155–162

Al-Izzi, S.A. and L.S. Al-Bassam, 1989. *In vitro* susceptibility of *Pseudomonas mallei* to antimicrobial agents. *Comp. Immunol. Microbiol. Infect. Dis.*, 12: 5–8

Andrew, J.M., 2001. BSAC standardized disc susceptibility testing method. *J. Antimicrob. Chemother.*, 48: 43–57

Ansabi, M. and M. Minou, 1951. Two cases of chronic human glanders treated with sulfamides. *Annl. Inst. Pasteur.*, 81: 98–102

Antonov, I.V., V.I. Iliukhin, L.D. Popovtseva and V.P. Batmanov, 1991. Sensitivity of Pseudomonas to currently used antibacterial drugs. *Antibiot Khimioter*, 36: 14–26

Batmanov, V.P., 1991. Sensitivity of *Pseudomonas mallei* to fluoroquinolones and their efficacy in experimental glanders. *Antibiot. Khimioter*, 36: 31–34

Batmanov, V.P., 1993. Treatment of experimental glanders with combination of sulfazine or sulfamonomethoxine with trimethoprim (in Russian). *Antibiot. Khimioter*, 38: 18–22

Burns, J.L., 2006. Antibiotic resistance of *Burkholderia* spp. *In*: Coenye, T. and P. Vandamme (eds.), *Burkholderia Molecular Microbiology and Genomic*, pp: 81–91. Horizon Bioscience, Wymondham, Norflok, UK

Burrow, G.E., R.J. Morton and W.H. Tales, 1993. Microdilution antimicrobial susceptibilities of selected gram-negative veterinary bacterial isolates. *J. Vet. Diag. Investig.*, 5: 541–547

Carlier, M.B., B. Scorneaux, A. Zenebergh, J.F. Desnottes and P.M. Tulkens, 1990. Cellular uptake, localization and activity of fluoroquinolones in uninfected and infected macrophages. *J. Antimicrob. Chemother.*, 26: 27–39

Dvorak, G.D. and A.R. Spickler, 2008. Glanders. *J. American Vet. Med. Assoc.*, 233: 570–577

Harland, D.N., E. Dassa, R.W. Titball, K.A. Brown and H.S. Atkins, 2007. ATP-binding cassatte systems in *Burkholderia pseudomallei* and *Burkholderia mallei*. *BMC Genomics*, 8: 83

Godoy, D., G. Randle, A.J. Simpson, D.M. Aanensen, T.L. Pitt, R. Kinoshita and B.G. Spratt, 2003. Multilocus sequence typing and evolutionary relationships among the causative agaents of melioidosis and glanders, Burkholderia pseudomallei and Burkholderia mallei. *J. Clin. Microbiol.*, 41: 2068–2079

Heine, H.S., M.J. England, D.M. Waag and W.R. Byrne, 2001. *In vitro* antibiotic susceptibilities of *Burkholderia mallei* (causative agent of glanders) determined by broth microdilution and E-test. *Antimicrob. Agents Chemother.*, 45: 2119–2121

Howe, C. and W.R. Miller, 1947. Human glanders: report of six cases. *Ann. Int. Med*., 26: 93–115

Kenny, D.J., P. Russell, D. Rogers, S.M. Eley and R.W. Titball, 1999. *In vitro* susceptibilities of Burkholderi mallei in comparison to those of other pathogenic Burkholderia spp. *Antimicrob. Agent Chemother.*, 43: 2773–2775

LiPuma, J.J., 2007. Update on *Burkholderia* nomenclature and resistance. *Clin. Microbiol. Newslett.*, 29: 65–69

Lopez, J., J. Copps, C. Wilhelmsen, R. Moore, J. Kubay, M. St-Jacques, S. Halayko, C. Kranendonk, S. Toback, D. DeShazer, D. L. Fritz, M. Tom and D.E. Woods, 2003. Characterization of experimental equine glanders. *Microb. Infect.,* 5: 1125–1131

Lozovaia, N.A., 1989. Sensitivity of Pseudomonas mallei to sulfanilamide combination *in vitro*. *Antibiot. Khimioter,* 34: 48–52

Manzeniuk, I.N., O. Manzeniuk, A.V. Filonov, I.G. Stepanshin and E.A. Svetoch, 1995. Resistance of Pseudomonas mallei to tetracyclines: assessment of the feasibility of chemotherapy. *Antibiot. Khimioter*, 40: 40–44

Mishankin, B.N. and A.M. Goldberg, 1969. Mechanism of natural penicillin resistance of inducers of glanders and melioidosis. *Antibiotiki*, 14: 1104–1106

Moore, R.H., D. DeShazer, S. Reckseidler, A. Weissman and D.E. Woods, 1999. Efflux-mediated aminoglycoside and macrolide resistance in Burkholderia pseudomallei. Antimicrob. *Agents Chemother.*, 43: 465–470

Muhammad, G., K.H. Hoblet, S. Bech-Nielsen, D.L. Jackwood and K.L. Smith, 1998a. Reproducibility of antibiogram of bovine mammary gland staphylococci under conditions of repeated subculturing. *J. Vet. Med.-B*, 45: 189–192

Muhammad, G., M.Z. Khan and M. Athar, 1998b. Clinico-microbiological and therapeutic aspects of glanders in equines. *J. Equine Sci.*, 9: 93–96

Naureen, A., M. Saqib, G. Muhammad, M.H. Hussain and M.N. Asi, 2007. Comparative evaluation of Rose Bengal plate agglutination test, mullein and some conventional serological tests for diagnosis of equine glanders. *J. Vet. Diag. Invest.,* 19: 362–367

Nemato, H., H. Yokobori and R. Azuma, 1961. Studies on treatment of antibiotics of experimental acute glanders in guinea pigs. *Bull. Natl. Inst. Anim. Health*, 41: 11–20.

OIE, 2008. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, 8th edition, pp: 919–928. Office International Des Epizooties, Paris

Papich, M.G. and J.E. Riviere, 2001. Chloramphenicol and derivatives. *In*: Richard, H. Adams (ed.), *Veterinary Pharmacology and Therapeutics,* 8th edition, pp: 868–876. Blackwell Publishing Co., Oxford, UK

Rotz, L.D., A.S. Khan, S.R. Lillibridge, S.M. Astroff and J.M. Hughes, 2002. Public health assessment of potential biological terrorism agents. *Emerg. Inf. Dis.,* 2: 225–230

Schoevers, E.J., L.A.M.G. Leengoed, J.H.M. Verheijden and T.A. Niewold, 1999. Effects of enrofloxacin on porcine phagocytic function. *Antimicrob. Agents Chemother*., 43: 2138–2141

Stepanshin, I.G., I.N. Manzeniuk, E.A. Svetoch and K.I. Volkovoi, 1994. *In vitro* development of fluoroquinolone resistance in glanders pathogen. *Antibiot. Khimioterap.*, 39: 30–33

Tezok, F., 1958. Three glanders strains isolated from three patients in 1956 and differing with regard to sensitivity to antibiotics. *Int. Congr. Microbial. Abstr*., VII: 344

Thibault, F.M., E. Hernandez, D.R. Vidal, M. Girardet and J.D. Cavallo, 2004. Antibiotic susceptibility of 65 isolates of *Burkholderia mallei* to 35 antimicrobial agents. *J. Antimicrob. Chemother.*, 54: 1134–1138

Tribuddharat, C., R.A. Moore, P. Baker and D.E. Woods, 2003. *Burkholderia pseudomallei* class a beta-lactamase mutations that confer selective resistance against ceftazidime or clavulanic acid inhibition. *Antimicrob. Agents Chemother*., 47: 2082–2087

Ulrich. R.K., M.P. Ulrich, M.A. Schell, H.S. Kim and D. DeShazer, 2006. Development of a polymerase chain reaction assay for the specific identification of *Burkholderia mallei* and differentiation from *Burkholderia pseudomallei* and other closely related Burkholderiaceae. *Diagnos. Microbial. Infect. Dis.*, 55: 37–45

Wuthiekanun, V., A.C. Cheng, W. Chierakul, P. Amornchai, D. Limmathurotsakull, W. Chaowagul, A. A.J.H. Simpson, J.M. Short, G. Wongsuvan, B. Maharjan, N.J. White and S.J. Peacock, 2005. Trimethoprim/sulfamethoxazole resistance in clinical isolates of *Burkholderia pseudomallei*. *J. Antimicrob. Chemother*., 55: 1029–1031

(Received 06 December 2008; Accepted 15 February 2010)