Int.J.Curr.Microbiol.App.Sci (2016) *5*(5): 342-349

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 5 (2016) pp. 342-349**
Journal homepage: http://www.ijcmas.com

Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.505.035

Generation and Characterization of Monoclonal Antibodies Specific to *Burkholderia mallei*

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Keywords

Burkholderia mallei, Glanders, monoclonal antibodies (MoAbs).

Article Info

Accepted:
15 April 2016
Available Online:
10 May 2016

A B S T R A C T

Glanders is a contagious disease of equines and is endemic in many countries. There are problems with existing glanders diagnostic tests and monoclonal antibody (MoAbs) based tests can be useful. The aim of this study was to generate MoAbs specific to *Burkholderia mallei*. BALB/c mice were used for the generation of MoAb and were injected with whole cell sonicated antigen (WCS) of *B. mallei*. After 3 injections, two final boosts were given and spleen cells of mouse showing the highest antibody titer to WCS antigen by ELISA collected and fused with myeloma cells in presence of PEG 2000. Twenty nine hybridomas producing antibodies were generated of which five stable clones were expanded, among them three clones were finally checked for the specificity of their antibodies against various *Burkholderia* species and other closely related bacteria. Two of the MoAbs DM01BUMA01 and DM01BUMA02 did not show any cross reaction with any of the tested bacteria, DM01BUMA03 cross reacted with *B. cenocepacia* and *B. gladoili*. The MoAbs can be utilized for detection of *B. mallei*.

Introduction

Burkholderia mallei is the causative agent of glanders. Glanders is a contagious and fatal disease of horses, donkeys and mules (OIE, 2010). The disease also has high zoonotic significance and *B. mallei* is listed biological warfare agent which requires handling in biosafety level-3 (BSL-3) laboratory (Wheelis, 1998). *B. mallei* causes nodules and ulcerations in the upper respiratory tract and lungs. A skin form also occurs, known as 'farcy'. Control of glanders requires testing of suspect clinical cases, screening of apparently normal equids, and elimination of positive reactors.

The disease has been eliminated from most countries of Western hemisphere through above mentioned countermeasures and trade restrictions. However, glanders is still endemic in Asia, Middle East, Central and South America. Recent outbreaks have occurred in many countries including India (Neubauer *et al.*, 2005; Malik *et al.*, 2012).

Clinical and bacteriological diagnosis of glanders is difficult in the early stages of the disease. Nearly 90% of infections exist as nonclinical or latent (Neubauer *et al.*, 1997). Various tests for diagnosis of glanders have been described e.g. BimA based ELISA,

complement fixation test (CFT), mallein test (Kumar *et al.,* 2011; Khan *et al.,* 2012). However, CFT remains one of important diagnostic tests and has been mandated by World Organization of Animal Health (OIE) as the confirmatory test for diagnosis of glanderous animal for international trade. The antibodies primarily directed against lipopolysaccharide (LPS) antigen are detected in CFT (OIE, 2010). Isolation of bacteria from lesions of diseased animal is difficult.

The monoclonal antibody (MoAb) generated by cell fusion between lymphocytes and myeloma cells is a proven useful reagent in detection and diagnosis of various diseases or their causative agents (Kohler and Milestien, 1975). Many biologists use the so-called MoAb shotgun approach, in which MoAbs are indiscriminately produced against crude immunogens, facilitating production of antibodies without any molecular information of the immunogens in advance.

Monoclonal antibody specific to *B. mallei* can be very useful in antigen based diagnosis of glanders or the detection of bacteria from non-clinical sources. Further, they can also be utilized in latex agglutination assay for quick detection of *B. mallei* culture. In this study, we report the generation and characterization of MoAb specific to *B. mallei.*

Materials and Methods

Bacterial Cultures and Growth Conditions

The bacterial cultures used in this study are listed in Table 1. The cultures were obtained from National Collection of Type Cultures (NCTC), Health Protection Agency, London; Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh or DRDE's cultures repository. *B. mallei* was grown in glycerol dextrose broth (GDB) containing peptone-10g, dextrose-10g, beef extract-5g, NaCl-5g, glycerol- 40ml in 1 liter of distilled water for 48 h at 37°C in a shaking incubator (150 rpm). Other bacteria were grown in the recommended media for 24h at 37°C under shaking conditions (150 rpm). The bacteria were enumerated by pour plate method. *B. mallei* and *B. pseudomallei* cultures were grown in BSL-3 laboratory.

Antigen Preparation

Three types of antigens were prepared- (a) whole cell sonicated (WCS) antigen: The bacterial pellet was harvested by centrifugation at 8000 rpm for 10 min followed by two washings and resuspension in phosphate-buffered saline (0.01 M PBS). The dissolved pellet was sonicated for 5 min (30 sec cycle on and 30 sec cycle off) followed by centrifugation at 8000 rpm for 10 min. The supernatant was collected and sterilized by filtration (0.45 µm syringe filter, Millipore). (b) Formalin inactivated cell (FIC) antigen: For preparation of formalin inactivated cells, 2.5% formalin (v/v) was added to the bacterial growth and kept overnight at 37°C. Overnight culture then centrifuged at 8000 rpm for 10 min, pellets were then washed, resuspended in equal volume of PBS. (c) Heat killed cell (HKC) antigen: The bacterial growth was kept at 80°C for 4h in shaking (100 rpm) water bath. The pellet was harvested by centrifugation at 8000 rpm for 10 min washed and resuspended in equal volume of PBS. To further ensure that the prepared antigen was sterile, 1ml of each type was allowed to grow in 10 ml of GDB or brain heart infusion (BHI) broth for 10 days at above mentioned conditions followed by plating on suitable plate medium.

Immunization

BALB/c mice were taken from Central Animal Facility of DRDE, Gwalior and were given water and food ad libitum. The mice were maintained and used in accordance with the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Forests and Environment, Govt. of India. The study had an approved animal protocol from Institutional Animal Ethics Committee of DRDE, Gwalior (protocol no. MB/15/51/SK). Five 6- 8 weeks old female mice were subcutaneously immunized with purified WCS of *B. mallei* strain NCTC 10229. Briefly, primary immunization was done with 25 µg of purified WCS in emulsion with Freund's complete adjuvant (Sigma, USA). Two booster doses of 25 µg protein with Freund's incomplete adjuvant (Sigma, USA) were followed on days 14 and 28. The antibody titer was determined by enzyme linked immunosorbent assay (ELISA) and mouse showing the highest titer was given 2 booster injections intraperitonially on consecutive days prior to sacrifice for generation of hybridomas.

Generation of Monoclonal Antibodies

Hybridoma fusions were performed to generate MoAbs, according to established procedures (Kohler and Milestien, 1975). Briefly, spleen cells from immunized BALB/c mice were fused with sp2/0-Ag.14-1 cells using 50% w/v polyethylene glycol (PEG) 2000 (Sigma Aldrich). Fused cells were grown in selective HAT-containing Dulbecco's modified Eagle's media (DMEM, Sigma) and 10% v/v fetal bovine serum (Sigma Aldrich). The generated hybrid clones were screened for their reactivity against the antigen by indirect ELISA. Promising hybridomas were further cloned by limiting dilution method in 96 well tissue culture plates (Nunc) and resulting specific hybridomas were subjected to expansion. Supernatant of wells containing hybrids were tested for the presence of antibodies against WCS antigen of *B. mallei* NCTC 10229 by ELISA. Consistently positive wells were cloned and subcloned thrice to obtain stable hybrids.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was used for screening of hybridomas, isotyping, to determine the specificity or detection limit of MoAb culture supernantant. For screening of hybridomas, ELISA modules (Nunc, maxisorp) were coated with WCS antigen (10ug/ml) in carbonate bicarbonate buffer (pH 9.6). The plate was incubated overnight at 4°C followed by blocking with 1% BSA at 37°C for 2h. This was followed by addition of antibodies (culture supernatant) and anti mouse IgG-HRP conjugate (1:4000) for 1h each. The plate was washed 5 times after each reaction with PBS-T (0.01 M PBS with 0.05% Tween-20) reagent. The ELISA was developed with o-phenylene-diamine dihydrochloride(OPD) in citrate phosphate buffer. The reaction was stopped with 2.5 N H_2SO_4 and absorbance quantified at 492 nm. For isotyping HRP-labeled goat anti-mouse IgG and IgM conjugates (BD Pharmingen) were used. The specificity of antibodies was checked against various bacteria (Table 1), which were added in 100 µl (10^7 cells/ml FIC and HKC or 10µg/ml WCS antigen) amount to the wells of ELISA modules (Nunc, maxisorp). Various antigen dilutions were used for determination of detection limit.

Immunobloting

WCS of standard strain *B. mallei* NCTC 10229 was prepared as described earlier and precipitated by 50% (1:4 v/v) TCA

overnight at 4°C. Next day precipitated WCS sample was centrifuged at 10,000 rpm for 15 min at 4°C and supernatant was discarded. The pellet was washed twice with cold acetone+0.07% BME, air dried and dissolved in SDS sample buffer. The antigen was resolved on 12% SDS-PAGE and transferred to nitrocellulose membrane by standard Western blotting procedure. DM01BUMA01, DM01BUMA02, and DM01BUMA03 culture supernatants were used as a primary antibody with the incubation of 1 h at room temperature on rocker followed by reaction with anti-mouse-HRP conjugate as secondary antibody with the same incubation as above. Finally reaction was developed using (DAB)-H_2O_2 as substrate.

Results and Discussion

B. mallei is a highly pathogenic bacterium of equines with high zoonotic potential. Many test methods have been described for diagnosis of glanders, of which CFT is mandated by OIE as confirmatory test for international trade. However, CFT has drawbacks in terms of sensitivity and specificity (Neubauer *et al.,* 2005). Further, tests are not available for direct detection of pathogen from clinical or non-clinical samples. In this scenario, MoAb based tests can be very useful.

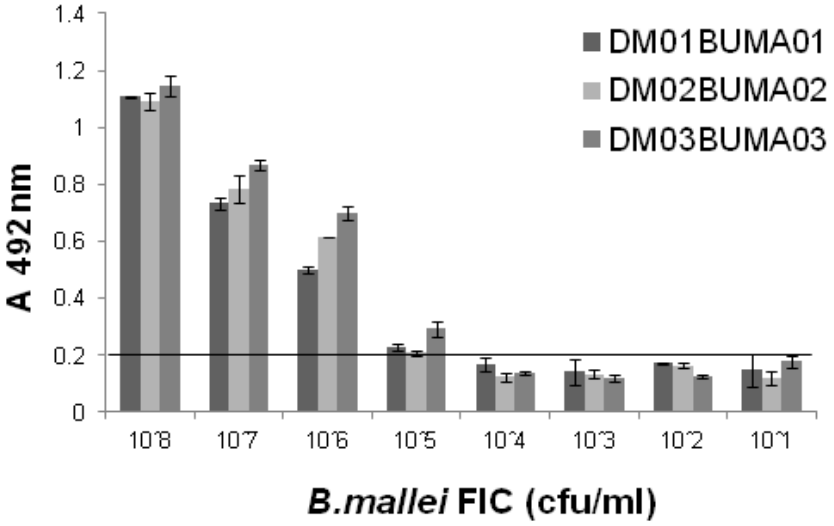
In the present work, efforts were made to generate MoAbs specific to *B. mallei*. Following fusion of sensitized spleen cells with myeloma cell line, 288 hybridomas were generated. These were screened for production of antibodies to WCS antigen and 29 of them were found to secrete antibodies. Five of these hybridomas were found to be stable and were expanded. However, two of the five clones did not react with FIC antigen of *B. mallei*, hence, were not taken further. Three type of antigens as described in the Materials and Methods were used. The idea of using FIC and HKC was that MoAb should be able to detect intact bacteria. Three clones were finally stabilized and preserved. The clones were designated as DM01BUMA01, DM01BUMA02 and DM01BUMA03. The isotyping of the generated MoAbs was determined by ELISA and DM01BUMA01, DM01BUMA02 and DM01BUMA03 were found to have IgG2b, IgG3 and IgG1 isotype respectively.

All three MoAbs were tested for specificity against different *Burkholderia* species and closely related bacteria by ELISA. The results are shown in Table 1. All MoAbs reacted with tested *B. mallei* strains and no cross reaction was observed with *B. pseudomallei* strains. Generation of MoAbs against *B. mallei* and *B. pseudomallei* has earlier been described (Feng *et al.,* 2006; Zao *et al.*, 2008; Duval *et al.,* 2014). Many of the generated MoAbs react with both the bacterial species and have been suggested to be more useful for passive protection (Jones *et al.,* 2002; Trevino *et al.,* 2006). It is understandable because, *B. mallei* is a deletion mutant of *B. pseudomallei* and the two bacteria are 99% identical at genomic level (Godoy *et al.,* 2003). Since the developed MoAbs are specific for *B. mallei*, these can be very useful for specific detection of *B. mallei*. The MoAbs except DM01BUMA03 also did not react with any other related *Burkholderia* or enterobacteria species.

Table.1 Specificity of MoAbs to various antigens of *Burkholderia* and related species

No.	Name of isolate	Type of antigen	DM01BUMA01	DM01BUMA02	DM01BUMA03
1.	*B. mallei* NCTC10229	WCS	+	+	+
		FIC	+	+	+
		HKC	+	+	+
2.	*B. mallei* NCTC 10247	WCS	+	+	+
		FIC	+	+	+
3.	*B. mallei* NCTC 10260	WCS	+	+	+
		FIC	+	+	+
4.	*B. mallei* NCTC 3709	WCS	+	+	+
		FIC	+	+	+
5.	*B. pseudomallei* NCTC 13392	WCS	-	-	-
		FIC	-	-	-
		HKC	-	-	-
6.	*B. pseudomallei* DB163BUPS32	WCS	-	-	-
		FIC	-	-	-
		HKC	-	-	-
7.	*B. pseudomallei* DB164BUPS33	WCS	-	-	-
		FIC	-	-	-
		HKC	-	-	-
8.	*Pseudomonas aeruginosa* MTCC 424	WCS	-	-	-
		FIC	-	-	-
		HKC	-	-	-
9.	*Pseudomonas putida* DB173PSPU1	WCS	-	-	-
		FIC	-	-	-
		HKC	-	-	-
10.	*B. cepacia* MTCC 438	HKC	-	-	-
11.	*B. cepacia* MTCC 1617	HKC	-	-	-
12.	*B. cenocepacia* 7656 DB174BUCE1	HKC	-	-	+
13.	*B. cenocepacia* DB175BUCE2	HKC	-	-	+
14.	*B. gladioli* MTCC 10216	HKC	-	-	+
15.	*B. glumae* MTCC 8496	HKC	-	-	-
16.	*Cupriavidus necator* MTCC 1472	HKC	-	-	-
17.	*Escherichia coli* MTCC 739	FIC	-	-	-
18.	*Salmonella typhi* DB177SAEN1	FIC	-	-	-
19.	*Salmonella paratyphi A* DB178SAEN2	FIC	-	-	-
20.	*Salmonella entritidis* DB179SAEN3	FIC	-	-	-
21.	*Klebsiella pneomoniae* MTCC 432	FIC	-	-	-
22.	*Yersinia enterocolitica* DB176YEEN1	FIC	-	-	-
23.	*Staphylococcus aureus* DB180STAU66	FIC	-	-	-

Fig.1 Detection limit of monoclonal antibodies as determined by ELISA. A-Formalin inactivated cells (FIC) of B- Heat killed cells of *B. mallei* were coated on the ELISA plate at various concentrations and detected by culture supernatant of MoAb. The horizontal line shows the cut-off value.



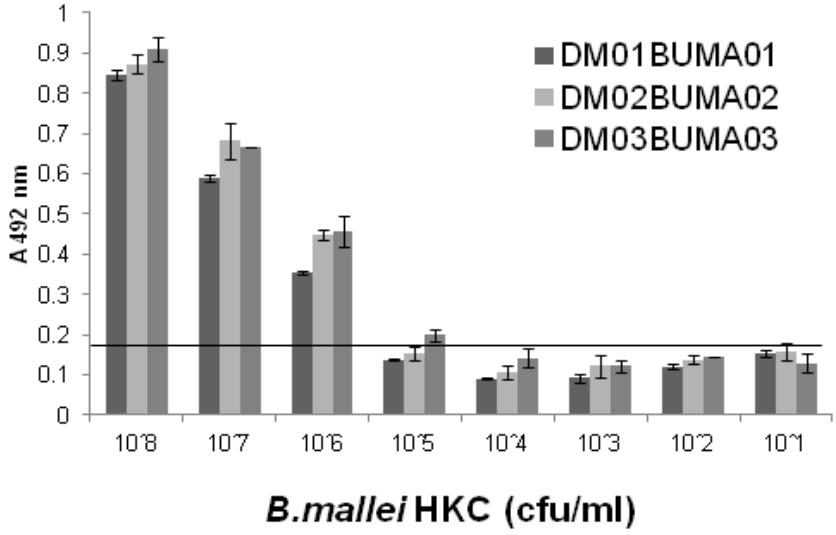
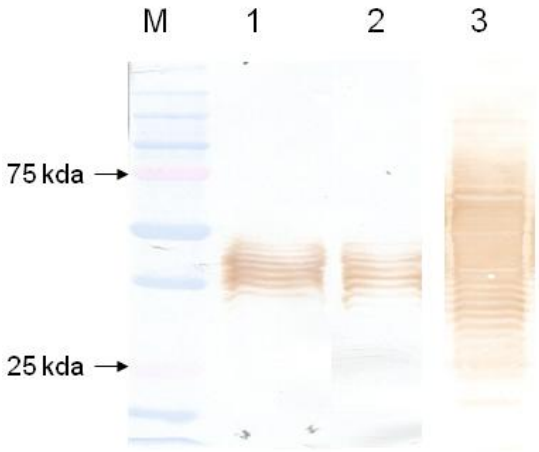


Fig.2 Western blot showing the reactivity of MoAbs with WCS antigen of *B. mallei*, M= protein marker, Lane 1: DM01BUMA01, Lane 2: DM01BUMA02, Lane 3: DM01BUMA03.



Detection limit of MoAb culture supernatant was determined by ELISA. All the MoAbs were found to detect 62.5 µg of WCS antigen (results not shown). Culture supernatant of DM01BUMA01 and DM01BUMA02, MoAbs could pick up 105FIC/ml or 10^6 HKC/ml of *B. mallei* DM01BUMA03 was a slightly better than other two MoAbs and gave higher OD in ELISA. The detection limit of DM01BUMA03 was 10^5 HKC or FIC/ml *B. mallei*. Results are shown in Fig. 1. The WCS antigen of *B. mallei* was allowed to react with MoAbs by immunoblotting. As expected all MoAbs reacted with WCS antigen (Fig. 2). A characteristic banding pattern of lipopolysaccharide (LPS) was observed especially with DM01BUMA03, suggesting that this antibody may be directed against LPS. The MoAbs described earlier by Feng *et al.*, 2006 also reacted with LPS.

In brief, MoAbs specific to *B. mallei* were generated in the present work. All of these are not cross reacting with closely related *B. pseudomallei* bacteria. The MoAbs can be utilized for specific detection of *B. mallei* in clinical or non-clinical settings

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How to cite this article:

Sudhir K. Dohre, Urmil Tuteja and Subodh Kumar. 2016. Generation and Characterization of Monoclonal Antibodies Specific to *Burkholderia mallei. Int.J.Curr.Microbiol.App.Sci.* 5(5): 342-349. doi: http://dx.doi.org/10.20546/ijcmas.2016.505.035