Comparison between Immunological and Molecular Based Methods for Diagnosis of *Mycobacterium* Infections in Cattle, Buffaloes and Human in Egypt

Elsayed MSA[1]*, Elkerdasy AF[2,3], Akeila MA[4] and Elsayed AA[5]

[1]*Department of Bacteriology, Mycology and Immunology, University of Sadat City, Minufyia, Egypt*
[2]*Department of Biochemistry, University of Sadat City, Minufia, Egypt*
[3]*Department of Biomedical Science, College of Pharmacy, Shaqra University, Al-Dawadmi, Saudi Arabia*
[4]*Department of Microbiology, Alexandria University, Egypt*
[5]*Department of Internal medicine and Animal Infectious Diseases, Cairo University, Egypt*

Abstract

Pathogenic mycobacteria are notorious for infections in animals and human their diagnosis hampered by atypical types. Animal products consumption is responsible for majority of diseased cases worldwide. Comparison between diagnostic tests appears to be lacking in Egypt. Therefore, this study aimed to evaluate diagnostic values of Flow-cytometry, Immuno-chromatography, Low-cost Density Microarray (LCD) array, High-Performance Liquid Chromatography (HPLC) and multiplex Polymerase Chain Reaction (PCR). Comparative Intradermal Tuberculin Test result was 1.31%. *M. bovis* and *M. kansasii* were high from animal samples while, *M. chelonae* and *M. malmoense* from human samples using LCD array, HPLC and multiplex PCR confirmation. Serological tests estimated with differences regarded to different antigens. Chembio DPP VetTB Assay gave the highest sensitivity result 94.8% while, TB-ST (Tuberkulose Schnell test) test kit showed the lowest 82%. Flow-cytometry, CD2, CD4, CD8 and δγ WC1+ cells were high in tuberculin-positive cases and low in negative proving pathogenic mycobacterial infections of tuberculin-positive cases. (LCD) array, (HPLC) and multiplex (PCR) proved sensitive discriminatory methods while; serologic assays and flow-cytometry represent rapid diagnostic tools. Further investigations required to improve the sensitivity and specificity of these tests. These results elucidate the importance of different diagnostic tests and considered backbone for future researches.

Introduction

Bovine tuberculosis is a paramount notifiable disease in dairy herds caused by *M. bovis*, crucial bacteria of wide host spectrum; affects many mammal species, humans, and wild animals those act as a reservoir and hamper its eradication [1-7]. It belongs to *Mycobacterium tuberculosis* Complex MTC which includes *M. tuberculosis*, *M. bovis, M. caprae*, *M. microti, M. canettii, M. pinnipedii,* **and** *M. africanum*. Perception of *M. bovis* pathogenesis elucidates its presence, propagation, and transmission [8-14]. Ante-mortem screening basically relies on tuberculin skin test, serologic tests, cytokine assays as indirect diagnostic methods. These tests may be limited by lack of species-specific reagents and validation, frequent captures to administer and record results, animal injuries (i.e., TST) and sample-handling requirements [15-18]. Although the sensitivity and specificity of serological assays may increase using specific antigens constituting alternative TB diagnostic tools [4]. Cell-Mediated immune response is the major host response to *mycobacterium* infections and is complicated by many significant differences. A common problematic theme is the prolonged latent asymptomatic period which can last for many years. Fundamentally, T cells are cells of major concern in this immune response. Centering upon T helper-1cells (Th-1) it plays vital role during latency that correlates to the control of pathogen proliferation and progression. Many studies focused on the protective role attributed to CD4, CD8, and γδ T cells [7] and CD2. Detection of changes in these cells considered as diagnostic markers for *mycobacterium* infection [15]. Moreover, Low-Cost and Density DNA microarrays (LCD) considered easy to use, sensitive and specific molecular tools for diagnosis of *mycobacterial* infections [6]. Furthermore, High-Performance Liquid Chromatography (HPLC) could be used for *mycobacterium* identification through analysis of mycolic acids with great specificity [10].

Hence, this study was planned to compare Flow-cytometry, Immuno-chromatography, Low-cost Density Microarray (LCD) and High-Performance Liquid Chromatography (HPLC) for rapid detection of *mycobacterium* in samples and isolates from cattle, buffaloes, and human cases.

Methodology

Tuberculin test

Comparative Intradermal Tuberculin Test of 6000 Holstein-Frisian cattle and native breed buffaloes. Briefly, after shaving two sites on one side of the neck (12 cm apart) and recording skin thickness initially, 0.1 ml of avian tuberculin PPD-A (Avian PPD 25,000 IU/ml) was injected intradermal into the upper site and an equivalent dose of (Mammalian or bovine PPD tuberculin: 2 mg/ml) injected into the lower site of the neck, 72 h post injection the skin thickness measured again and recorded. The corresponding thickness ≥1 mm to <4 mm, ≥1 mm to <3 mm, and ≥1 mm to <2 mm classified as doubtful result. The result considered negative if skin response <1 mm and positive if skin fold thickness of ≥4 mm [2].

Sampling and Cultivation

Animal samples: Blood samples collected from 120 cattle and

Product Code	Ig isotype	Specificity
MCA1653F	IgG2a	CD4
MCA837F	IgG2a	CD8 CD8 alpha
MCA838G	IgG2a	WC1+ δγ TCR1 chain
MCA833F	IgG1	CD2

Table 1: Utilized monoclonal antibodies.

buffaloes (79 tuberculin positive, 41 tuberculin negative). 10 ml blood in sterile McCartney tubes for serum, 37 ml mixed with 12.5 ml acid citrate dextrose (anticoagulant) for lymphocytes separation. Tuberculin positive slaughtered (according to Egyptian law) then subjected to postmortem examination. Mandibular, mediastinal, left and right bronchial, hepatic and mesenteric lymph nodes were collected. Tissue samples from lungs, liver, spleen and intestine as well. Samples classified to the visible lesion (VL) and nonvisible lesion (NVL) then processing for cultivation [16-20] as well as for DNA extraction after concentration.

Human sputum samples from diagnosed cases: Three first morning sputum specimens of 5-10 ml after a deep productive cough for three consecutive days into a sterile cap. The specimens kept at 4°C before processing with N-acetyl-L-cysteine-NaOH (NALC-NaOH).

Cultivation: Animal samples cultivated onto Modified Lowenstein -Jensen pyruvate media (LJ-P) while, human samples cultivated onto Modified Lowenstein -Jensen glycerol media (LJ-G) [19-25].

DNA extraction and molecular examination by LCD array

QIAamp DNA extraction Mini prep Kit for extraction of DNA. Molecular typing and LCD array performed according to [6]. Confirmation of the *M. bovis* by multiplex Polymerase Chain Reaction using primers targeting Miru [26], VNTR 3232, Miru [24], QUB 4156c and QUB 1451 [26].

Flow-cytometry

Animal blood sampling for Flow-cytometry: 1st group of 4 Holstein Frisian cattle (tuberculin positive), 2end control group of 4 Holstein Frisian cattle (tuberculin negative). 3rd group 4 native breed buffalos (tuberculin positive) and the 4th control group of 4 native breed buffalo (tuberculin negative).

Direct Immunofluorescence Staining according to Bio-Rad AbD Serotec protocol

Blood lysis by adding 2 ml freshly prepared erythrolyse red cell lysing buffer and mix well. Incubate for 10 minutes at room temperature. Centrifuge at 300-400 g for 5 minutes at room temperature and discard the supernatant. Wash with 2 ml room temperature PBS/BSA, centrifuge at 300-400 g for 5 minutes and discard the supernatant. Adjust the cell suspension to a concentration of 1×10^7 cells/ml with cold (4°C) PBS/BSA buffer. Aliquot 100 μl of the cell suspension into as many test tubes as required. Add antibody at the vendor-recommended dilution. Mix well and incubate at 4°C for at least 30 minutes, avoiding direct light. Wash cells with 2 ml cold (4°C) PBS/BSA, centrifuge at 300-400 g for 5 minutes at 4°C, and discard the resulting supernatant. Resuspend cells in with 200 μl of 0.5% paraformaldehyde in PBS. The cells were analyzed using Becton Dickinson FACS Caliber flow cytometer equipped with argon and red lasers. This machine connected to Macintosh 5 computer, with Cell Quest software (Becton Dickinson Immunochemistry systems, San Jose CA) at the Flow-cytometry Unit, Department of Clinical Pathology, Faculty of Medicine, Minufyia University. FCS express software (De Novo software, Thornton, Ontario) used to analyze the data (Table 1).

Serological testing and Immunochromatography

Using Acon rapid test for diagnosis of *M. tuberculosis.* Chembio DPP Vet TB test for *M. tuberculosis* and *M. bovis.* TB-ST (Tuberkulose Schnelltest) test Lionex diagnostics and therapeutics GmbH and *Diavita tuberculosis* rapid test according to the manufacturer instructions.

High-performance liquid chromatography (HPLC)

The HPLC performed after LCD array for confirmation. Bacterial growth in LJ medium collected by a swab and saponified with 2 ml KOH 25% in methanol: H_2O (v:v). Autoclaved for 1 h at 121°C, 15 psi, to cleave the mycolic acids bound to the cell wall and HPLC fulfilled according to [8] Reproducible chromatographic diagnostic peaks using reference strains (*M. bovis* ATCC 19210, *M. chelonae* ATCC 35752, *M. kansasii*, ATCC 12478, *M. malmoense* ATCC 29571, *M. tuberculosis* ATCC 25177). HPLC System Agilent 1100 Series used.

Data Analysis

Data analyzed by Statistical Analysis System software package SAS for Windows, version 8 (SAS Institute, Cary, NC). Independent t-test used and the significant differences between flow-cytometer means represented by Mean ± SE and comparison between data to detect significant difference between that of tuberculin positive and negative cases.

Results

Ante-mortem intradermal testing and slaughter house examination

There found low proportions of tuberculin-positive cases which was detected by comparative tuberculin testing 79/6000 (1.31%). Significant difference was found between VL and NVL (<0.0001) (Table 2).

Isolation and identification of *mycobacterium* species from various lesions

There located weak downhill (negative) linear relationship (r): -0.173 between animal lesions, culture, and microscopy. It was found that only 51/89 (57.3%) were positive for isolation due to the absence of isolation from calcified pulmonary and digestive lesions and diminished isolation from NVL. On the other hand, human samples gave highest isolation 10/10 (100%) from (Table 3).

Molecular identification of *mycobacterium* isolates by LCD array, HPLC and multiplex PCR

M. bovis represented 20 (80%) and 3 (42.9%) from VL and NVL isolates for *M. kansasii* only 4 (16%) from VL. *M. chelonae* and *M. malmoense* expressed 2 (20%) and 1(10%). Perfect uphill (positive) linear relationship (r): +1 between LCD array on tissue and corresponding isolates added to that, between LCD array, HPLC

Place of collected samples	No. of animals	Tuberculin +ve		PM finding			
				VL		NVL	
		No	%	No	%	No	%
Different localities at Alexandria, El-Behira, El-Gharbia, Kafr El-Sheikh and Menofyia Governorate (within two years)	Cattle and buffaloes	6000	1.31%	60	75.94%	19	24.05%

Table 2: Results of tuberculin test and post mortem examination. (Significant difference between VL and NVL is <0.0001).
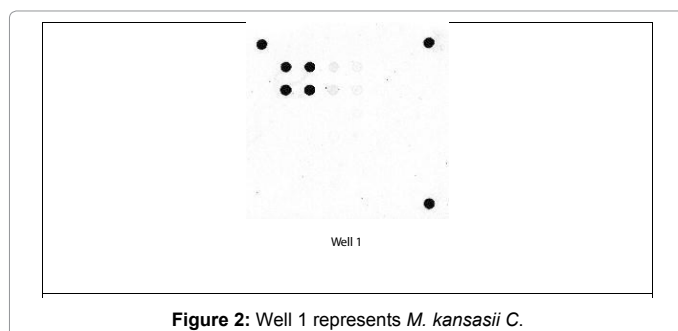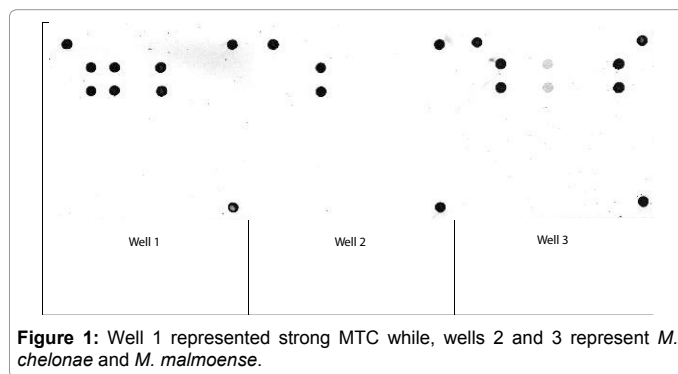
Post-mortem findings	No. of samples	Culture and microscopy	
		No	%
Generalized (VL)	7	7	100%
Localized head lymph nodes (VL)	8	7	87.5%
Pulmonary calcified (VL)	15	-ve	0.0%
Pulmonary uncalcified (VL)	8	8	100%
Digestive (VL)	10	-ve	0.0%
Mixed (VL)	12	12	100%
Non Visible Lesion (NVL)	19	7	15.78%
Total	89	51	57.3%
Human	10	10	100%

Table 3: Results of bacteriological examination of animal and human samples. [Weak downhill (negative) linear relationship (r) = 0.173 between collected samples isolation and microscopy].

Type of samples	No. of isolates	The result of LCD array		The result of HPLC on culture	The multiplex PCR
		Tissue	Culture		
Cattle and buffaloes VL.	25	20 (80%) MTC	20 (80%) MTC.	20 (80%) MTC.	20 (80%) *M. bovis*
		4(16%) *M. chelonae*	4(16%) *M. kansasii.*	4 (16%) *M. kansasii*	Not performed
		1 (4%)*	1 (4%)*	1 (4%) No similarity	Not performed
Cattle and buffaloes VL.	7	3 (42.9%) MTC	3 (42.9%) MTC	3 (42.9%) MTC	3 (42.9%) *M. bovis*
		4 (57.1%)*	4 (57.1%)*	4 (57.1%) No similarity	Not performed
Human samples.	10	2 (20%) *M. chelonae*		2 (20%) *M. chelonae*	Not performed
		2 (20%) *		2 (20%) No similarity	Not performed
		1(10%) *M. malmoense*		1 (10%) *M. malmoense*	Not performed
		5 (50%) Negative		5 (50%) Negative	Not performed

Untypable (means got mycobacterium DNA but not similar to LCD patterns, HPLC no similarity (means gave curve but not similar to listed species). Perfect uphill (positive) linear relationship (r): +1 between LCD array on tissue and corresponding isolates and between LCD array and HPLC.

Table 4: Molecular identification by LCD array, HPLC and multiplex PCR confirmation of *Mycobacterium tuberculosis* complex.

Figure 1: Well 1 represented strong MTC while, wells 2 and 3 represent *M. chelonae* and *M. malmoense*.

Figure 2: Well 1 represents *M. kansasii C*.

and multiplex PCR (Table 4), (Figures 1 and 2) Chromatograms (1, 2, 3 and 4).

Untypable (means got mycobacterium DNA but not similar to LCD patterns, HPLC no similarity (means gave curve but not similar

to listed species). Perfect uphill (positive) linear relationship (r): +1 between LCD array on tissue and corresponding isolates and between LCD array and HPLC.

Clinical evaluation of sensitivity, specificity, positive and negative predictive values of antigen rapid TB test kits

Chembio DPP VetTB Assay gave the highest sensitivity results with 94.8% while, TB-ST (Tuberkulose Schnelltest) test kit was the lowest with 82%. Furthermore, Acon test gave the highest specificity 96% and Chembio DPP VetTB Assay was the lowest 88.9% (Table 5).

Flow-cytometric evaluation of tuberculin positive and negative animals proved a noticeable increase in tuberculin-positive cattle. CD2+ cells soared over than 3 times, CD4 was twice that of tuberculin-negative cattle. Moreover, from tuberculin positive buffaloes CD2+ and CD8 were twice more the counts of their counterparts from tuberculin negative buffaloes.
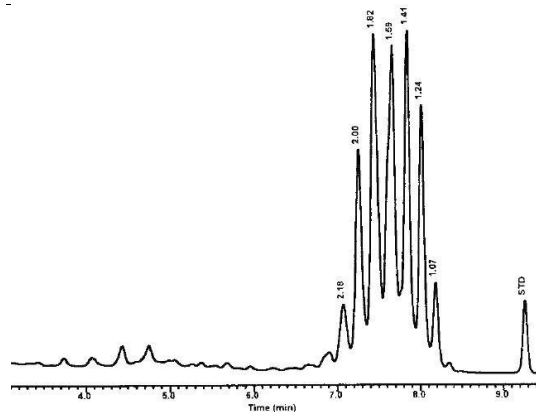
Significant difference ($P < 0.0001$) present between the counts of CD2+, CD4, CD8 and WC1+δγ of tuberculin-positive from counts of tuberculin- negative animals (Table 6).
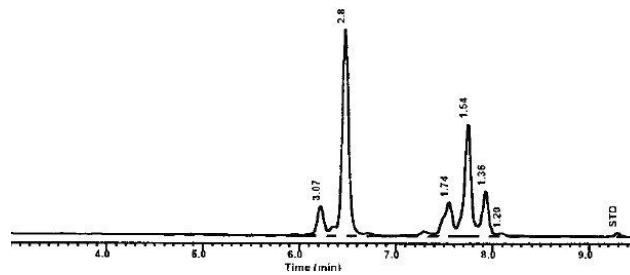
Discussion

M. bovis is a crucial infectious agent of wide host spectrum besides cattle it affects other mammal species including humans. It belongs to *Mycobacterium tuberculosis* Complex members [14-16]. Interestingly, the overall incidence level of *Mycobacterium* in Table 2 was 1.31% lower than 2015 records from Egypt [23]. On the contrary, nearly similar to obtained results at Mozambique [27]. This low percentage attributed to regular commitment with test and slaughter program of the authorities to control the spread of infections.
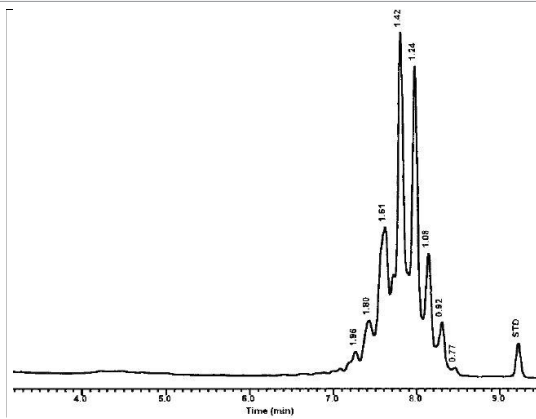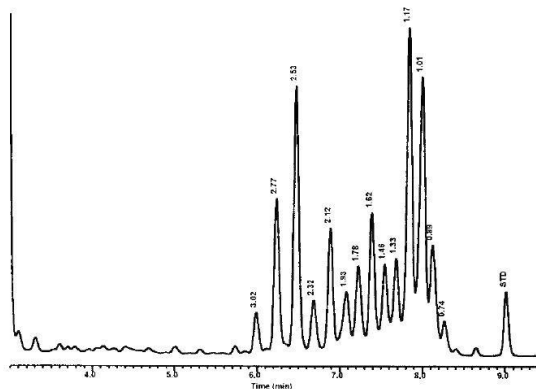
Chromatogram 1: *M. bovis.*

Chromatogram 2: *M. kansasii.*

Chromatogram 3: *M. chelonae.*

Chromatogram 4: *M. malmoense.*

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Type of serological test	Acon test	Chembio DPP VetTB Assay	TB-ST (Tuberkulose Schnell test) test kits
Sensitivity	93%	94.8%	82%
Specificity	96%	88.9%	92.5%
Positive Predictive Value	28%	34%	12%
Negative Predictive Value	100%	100%	100%

Table 5: Results of rapid bovine TB serological tests. Sensitivity, specificity, positive predictive and negative predictive were calculated against the isolation as gold standard.

Animal type	CD4	CD8	WC1+δγ	CD2+
Tuberculin positive (4) cattle	3400 ± 173	2150 ± 663	1350 ± 144	3900 ± 692
Tuberculin negative (4) cattle	1700 ± 404	1950 ± 144	850 ± 144	1150 ± 28
Tuberculin positive (4) buffaloes	2525 ± 441	0122 ± 270	2542 ± 222	5444 ± 8221
Tuberculin negative (4) buffaloes	2522 ± 884	8922 ± 525	2275 ± 092	2242 ± 626

Table 6: Flow-cytometry results of direct labeling of CD2+, CD4, CD8 and WC1+δγ cells. (Significant difference between cells of tuberculin positive and negative animals is $P < 0.05$).

Notably, the obtained VL and NVL results were analogous to that from Ireland 2013 [25]. In comparison with recent records from Egypt the VL was higher than 2015 records [23] while the NVL lower than these records. Significant difference was found between VL and NVL (<0.0001) that considered a feature of *M. bovis* infections that cause variation in the shape and distribution of lesions with respect to the isolation rate 57.3% from Table 3 nearly like that from cattle in Ethiopia after 2010 data [3]. The obtained isolation result from human sputum 10/10 (100%) was higher than the 2012 records from Egypt [21].

Isolation and identification of Mycobacterium species from various lesions in different organs

This mainly reflects various routs of infection and even possible secondary spread within the animal body.

Identifying *Mycobacterium* species using traditional methods is time consuming and expensive. For this reason, LCD array, HPLC and multiplex PCR confirmation performed here as attractive alternatives to the most traditional techniques. That's mainly due to the high sensitivity and specificity of these diagnostic techniques:

From the results of molecular identification of VL tissue samples and culture (Table 4), Figures 1 and 2 and chromatogram 1, it was found that 20/25 (80%) confirmed to be from MTC members this result comes consistent with results from Brazil during 2014 [1].

And comparing the results from VL and NVL to data from the USA in 2012 [9], the isolation was lower, molecular identification results of MTC were higher while, that for the atypical was lower.

The incidence of non-tuberculous *mycobacteria* (NTM) infections has increased over the past couple of decades after reports from Canada [5]. Concerning molecular confirmation of results of *M. kansasii* Table 4, Figure 2 and chromatogram 2 from VL tissue and culture was 4/25 (16%) higher than results reported in USA from 2004 to 2011 [28] But comport with him in the possibility to isolate non-tuberculous mycobacteria from gross tuberculous lesions.

Over and above, the results of molecular identification from NVL tissue samples and culture positive samples Table 4 proved that 3/7 (42.9%) belong to MTC members. The untypable isolates were 4/7 (57.1%) lower than results obtained from Project SE3262), funded by Department of Environment, Food and Rural Affairs, UK (www.defra. gov.uk) [25].

From Table 4, Figure 1 and chromatogram 3, the molecular confirmation of mycobacterial DNA from human samples and isolates *M. chelonae* represented 2/10 (20%) comply with data reported from northeastern USA [24].

Moreover, only 1/10 (10%) (Table 4), Figure 1 and chromatogram 4 proved *M. malmoense* which confirmed by reports from Canada [5] Great correlation expressed between LCD array, HPLC and multiplex PCR as stated after results from Texas Department of Health USA [11]. *M. bovis* was most often isolated from cattle and buffaloes besides atypical species as *M. kansasii*. *M. chelonae and M. malmoense* reported from human cases with no evidence of pathogenic *mycobacterium* infection.

From Table 5, sensitivity and specificity of Acon test nearly comparable to results from Pakistan [12]. The results of DPP VetTB assay and TB-ST (Tuberkulose Schnell test) higher than published data from National Animal Disease Center USA, and Brazil [17-22], the variability in readings of different serological tests could be attributed to the stage of *mycobacterium* infection and the different antigens contained in each test.

With regard to data obtained in Table 6, it was trenchant that CD2, CD4, CD8 and δγ WC1+ from *M. bovis* infected cattle and buffaloes higher than tuberculin negative cases. This basically confirms infection with pathogenic *mycobacterium* especially *M. bovis* and comes in agreement with results gained from the UK and USA [13-29]. This evidence indicates in vivo activation of these populations and great probability of being a diagnostic markers of pathogenic *mycobacterium* infection.

Conclusion

This is the first report in Egypt about comparing different diagnostic techniques to detect *Mycobacterium* infections. It is clear that lower rates of pathogenic *Mycobacterium* species present in Egypt. But, still the low sensitivity of tuberculin test constituting a nightmare which calls for finding rapid, sensitive and specific ante-mortem diagnostic tool. Although (LCD) array, (HPLC) and multiplex (PCR) proved great sensitivity they override culture in being low time consuming methods. Serological tests and flow-cytometry are promising but further investigations using different antigens in different stages of infection are required.

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