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Detection of anti-*Mycobacterium avium* subspecies *paratuberculosis* antibodies in thyroid and type-1 diabetes patients

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Mycobacterium avium subspecies *paratuberculosis* (MAP) causes granulomatous intestinal disease in animals (Johne's diseases). MAP has also been associated with several autoimmune disorders. In this study, we screened serum samples from confirmed patients of thyroid and type 1 diabetes for the presence of antibody against MAP. We used newly developed 'cocktail ELISA' (based on recombinant secretary proteins) and extensively validated 'indigenous ELISA' (based on whole cell protoplasmic antigen) and both the tests were also compared for their diagnostic potential. A total of 90 serums samples were included of which anti-MAP antibodies was detected in 28.8% and 26.6% of samples by indigenous ELISA (iELISA) and cocktail ELISA (cELISA), respectively. There was almost perfect agreement between the two tests in detecting the anti-MAP antibodies. Study raises concern on high detection of anti-MAP antibodies in human, thus warranting necessary control measure to minimize MAP exposure in human beings.

Keywords: *Mycobacterium avium* subspecies *paratuberculosis*, type 1 diabetes, thyroid, cocktail & indigenous ELISA.

Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP), the causative agent of Johne's disease (JD) in domestic livestock species, has wide host range. MAP infection has been reported in wild animal species, primates and also in humans[1-2]. MAP has been strongly associated with Crohn's disease (CD) in humans due to similar pathology, isolation of MAP isolate and other supportive evidences[3]. In addition to CD, MAP has also been implicated in multiple diseases like type 1 diabetes, Hashimoto's thyroiditis, multiple sclerosis, sarcoidosis, Blau syndrome etc[4].

Early and accurate diagnosis is key element in control of JD, however due to absence of dedicated control programme in India and variable sensitivity and specificity of available diagnostics it remains difficult to restrict the transmission of MAP. enzyme linked immunosorbent assay (ELISA) is one of the most frequently used screening tests used for the detection of MAP infection, however, variable sensitivity and specificity of available commercial diagnostics is an important issue. Earlier, we have developed indigenous ELISA (iELISA) using whole cell protoplasmic antigen from native strain of MAP'S 5' Indian bison type. Although, iELISA showed superior diagnostic value as compared to commercial ELISA[5], still there is a scope of further improving the sensitivity and specificity of test.

Secreted antigens have long been acknowledged to play central roles in bacterial-host interactions and it can serve as markers for early diagnosis of disease[6]. Previous study demonstrated increased sero-reactivity of secretary proteins as compared to other cellular proteins in MAP-infected animals[7]. Thus, use of secretary antigens may increase the sensitivity of ELISA substantially for sub-clinically infected cattle. Recently we developed new cocktail ELISA (cELISA) test by using six sero-reactive secretary proteins (1693c, 2168c, ModD, 85C, Pep AN and Pep AC). In our previous studies, cELISA was found sensitive to detect the MAP infection and also had potential to differentiate between infected and vaccinated animals (DIVA)[8]. Here, we aim to compare the diagnostic potential of both cELISA against iELISA in detecting anti-MAP antibodies in patients suffering from thyroid and type 1 diabetes.

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Materials and Methods

Ethics

This study was undertaken as part of a multi-centric project (outreach project on zoonotic diseases) granted by Indian Council of Medical Research (ICMR), New Delhi. The study was approved by Ethical Committee of National JALMA Institute, Agra (a collaborating institute for the same project).

Sample and ELISA Testing

A total of 90 blood samples from confirmed cases of thyroid and type-1 diabetes patients were included in this study. Serum from blood samples was harvested and stored at -20^{o}C until tested by ELISA. Each samples were tested using cELISA and iELISA as per method described earlier[8-9]. We used known positive (showing high titer for anti-MAP antibodies) and negative human sera as control for calculating and interpreting the test result.

Test Result Interpretation and Statistical Analysis

Optical densities (OD) of samples were transformed and expressed as sample-to-positive (S/P) ratios[10]. Based on calculated S/P ratio, samples were categorized as negative (N; 0.00-0.09), suspected (S; 0.1-0.24), low positive (LP; 0.25-0.39), positive (P; 0.4-0.99) and strong positive (SP; 1.0-10.0). To achieve meaningful statistical outcome and also in view of high mycobacterial exposure among population (due to BCG vaccination, high TB burden and frequent presence of mycobacteria in environment), samples with high S/P ratio were only considered as 'positive'. Therefore, samples with S/P ratio ≥ 0.40 (P+SP) were considered as 'positive' and samples with S/P ratio 0.00-0.39 (N+S+LP) were considered as 'negative'.

Considering iELISA as standard method, percent sensitivity of cELISA was determined. Additionally, proportional agreement and statistical difference between the two tests were also analyzed using kappa statistics and McNemar's calculation, respectively (GraphPad software, USA).

Results

Our study showed the presence of anti-MAP antibody among patients with thyroid/diabetes. Of total 90 samples tested, 26 (28.8%) and 24 (26.7%) samples were detected as positive by iELISA and cELISA, respectively. As compared to cELISA, iELISA detected 2 additional samples as positive. However, profiling in context to S/P ratio was better in cELISA. Of the 24 samples falling in category of positive (P) by iELISA, 7 were grouped into strong positive (SP) by cELISA. Similarly, samples classified under low positive (LP), suspected (S) and negative (N) by iELISA were differentially classified by cELISA. Samples stratified into different categories of S/P ratio by both the ELISA tests are depicted in Table 1 and Figure 1.

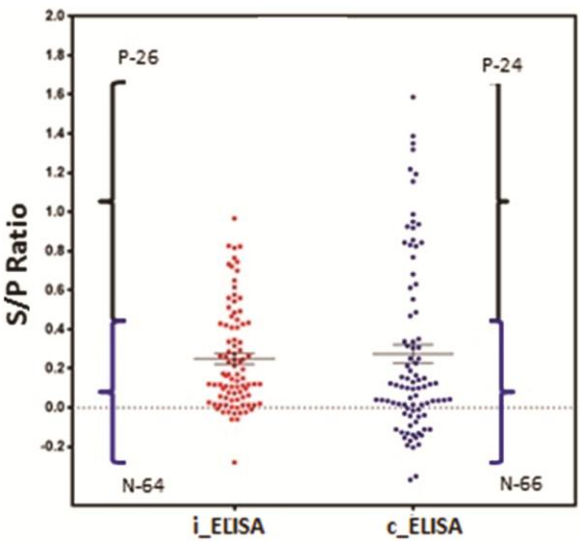

Fig. 1 — S/P ratio based profiling of samples tested by iELISA and cELISA using scattered plot analysis. (Abbreviation: S/P- sample to positive; P- positive; N- negative)

Table 1 — Sero-status of patients (n = 90) tested for anti-MAP antibodies using iELISA and cELISA

			cELISA *n* (%)				
	iELISA *n* (%)		Positive; 24 (26.7)		Negative; 66 (73.3)		
			SP	P	LP	S	N
			7 (7.8)	17 (18.9)	7 (7.8)	16 (17.8)	43 (47.8)
Positive 26 (28.9)	SP	0 (0.0)	-	-	-	-	-
	P	26 (28.8)	7	17	2	-	-
Negative 64 (71.1)	LP	10 (11.1)	-	-	4	3	3
	S	23 (25.5)	-	-	1	8	14
	N	31 (34.4)	-	-	-	5	26

LP- low positive; S- suspected; N- negative; P- positive; SP- strong positive

Proportional agreement between both the tests to detect anti-MAP antibodies was 97.8% (kappa score= 0.945; CI=0.869 to 1.000), thus showing a perfect agreement. The analytical difference between the two ELISA tests was non-significant (p=0.7392).

Discussion

MAP is endemic among domestic livestock in India. Since MAP is excreted through milk of infected animals, it is therefore continuously entering into human food chain. In recent past, sufficient evidence has been obtained to clearly show that many patients with auto-immune disorders, especially Crohn's disease are infected with MAP[11]. MAP has also been implicated in type 1 diabetes as a putative environmental agent triggering or accelerating the disease and also in Hashimoto's thyroiditis which is a common cause of hypothyroidism[12]. Our study reported high presence of anti-MAP antibodies among patients suffering from either thyroid disorder or type 1 diabetes. In agreement with our observation, high presence of anti-MAP antibodies has also been reported earlier in Iranian diabetes patients[13]. Our study has limitation as we could not collect patient history/demographics due to limited accesses with patients.

MAP is a major threat for both livestock and human population in this century. Using semi-purified protoplasmic antigen of goat origin harvested from 'Indian bison type' biotype of MAP (strain 'S5') based 'iELISA' showed significantly higher sensitivity as compared to commercially available ELISA kits[14-18]. Indigenous ELISA correlated well with culture and performed as good screening test for domestic livestock[19] and human population as well[20]. In our earlier study, we evaluated the diagnostic efficacy of iELISA in culture positive CD patients. All culture positive CD cases showed positive sero-status in iELISA, thus indicating iELISA as sensitive and accurate to detect MAP infection[20]. In present study, our newly developed cELISA also showed sensitivity close to iELISA. On analyzing the data as per different categories of S/P ratio, it is observed that out of 43 samples classified by cELISA as negative, 14 were borderline and 3 were in low positive category. Thus, indicating more sensitive or have better profiling of infection stage by cELISA. As another limitation of study, the precise sensitivity and specificity of test could not be assessed due to non-inclusion of any gold standard test. Culture, though considered as gold standard test but its sensitivity and specificity also get compromised due to progressive nature of disease (which involves different immunological mechanism in early, sub-clinical and clinical stages) and intermittent shedding of bacteria in clinical samples (feces and milk). Moreover, culture has disadvantage in term of long turn-around time (about 2-3 months) to get the result. Therefore, immunological test like ELISA seems as promising screening tool for MAP infection.

Although not investigated, cELISA may have higher specificity than iELISA since only few selected antigenic proteins was used that obviously minimize the cross-reactivity. This assumption is well supported by our previous study in which cELISA showed differentially high titer among infected animals as compared titer detected among vaccinated animals. Considering this important differential diagnostic value and high sensitivity in detecting anti-MAP antibodies in animal and humans, cELISA deserve to be further evaluated and explored for future control program.

Conclusions

This study demonstrated utility of ELISA test in screening of MAP infection. Both, whole cell protoplasmic and secretary antigens had similar diagnostic potential in detecting anti-MAP antibodies. High presence of anti-MAP antibodies warrant further investigation on the role of MAP in precipitation and progression of thyroiditis and type 1 diabetes. Furthermore, necessary action needs to be taken to minimize the exposure of MAP to human beings.

Conflict of Interest

The authors declare that they have no conflict of Interest.

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