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Some Observations on the Performance of the Liquid-Phase Blocking Sandwich ELISA for Detection of Foot and Mouth Disease Virus Antibodies

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ملخص لبحث

تم إختبار مائة عينة مصلية من الأبقار السودانية بواسطة المقايسه المناعيه المرتبطه بالانزيم ذات الطور السائل والحاصرة (Liquid-Phase Blocking ELISA) للاضداد النوعية للنوعين المصليين "O" و "SAT1" من فيروس الحمى القلاعية وذلك باستعمال تراكيز مختلفة من المستضدين " O" و "SAT1" و مستحضرات الاختبار التى تم تحضيرها في المعمل المرجعي للحمى القلاعية ببربرايت (المملكة المتحدة) .

أظهرت الدراسة أن تركيز نسبة المستضد (من مستحضرات الإختبار) إلي الأضداد النوعية (في العينة المصلية) إضافة الي الشراهة (Avidity) المصلية هما أكثر العوامل تأثيراً علي قدرة التقنية المذكورة في الكشف عن الأضداد النوعية في العينة المصلية. وجدت أن أقل مقدرة لقيم التقنية عندما كانت نسبة المستضد الى الأضداد النوعية متعادلة و أعلى قيم عندما مالت النسبة لجانب المستضد . كانت العلاقة التلازمية (Correlation) بين الأمصال المرجعية الموجبة (إصابة تجريبية) و العينات المصلية الموجبة (إصابة طبيعية) اضعف ما تكون عند إستعمال تراكيز منخفضة من المستضد.

إستنتجت الدراسة أن زيادة نسبة المستضد تزيد من مقدرة التقنية علي الكشف عن الأضداد النوعية وذلك لأن المركبات المناعية (Immune Complexes) يمكن حصرها (blocking) بكفاءة أعلى في حالة زيادة نسبة المستضد أكثر مما في حالة تعادل المستضد و الأضداد النوعية(في العينة المصلية) كما أن الأضداد النوعية ذات الألفة (Affinity) العالية في الأمصال ضعيفة الإيجابية تتفاعل بكفاءة أكبر عند زيادة نسبة المستضد . استنتجت الدراسة أيضاً أن زيادة تركيز المستضد إلى أقل من ضعف الجرعة المقررة و ذلك في حدود الكثافة الضوئية (Optical Density) المطلوبة 1.9 تزيد من حساسية التقنية إلي درجات مرغوبة.

Summary

One-hundred Sudanese cattle sera were screened for foot and mouth disease (FMD) serotypes "O" and "SAT1" antibodies by the liquid-phase blocking ELISA (LPBE) using different antigen concentrations, and reagents prepared by The World Reference Laboratory (WRL) for FMD. It was evident that, not only the concentration of antigen but the ratio of antigen to antibodies as well as antibody nature (avidity) are the most important determining factors in detection of serum blocking activity.

Lowest percentage inhibition (PI) values for a given serum were detected when antigen and antibodies were expected to be about equal,

and highest values were detected when antigen was in excess. Poor correlation between positive reference antisera (experimental infection) and positive test sera (natural exposure) was particularly evident when relatively low concentrations of antigen were used. It is concluded that increase in antigen concentration increases the detected specific blocking activity as excess-antigen immune complexes are more efficiently blocked than equivalent-antigen immune complexes, and high avidity antibodies, in weak positive sera, react preferentially. Antigen concentrations that represented less than two fold increase of the pre-estimated dose and that gave OD values well below the predefined limit of 1.9, increased sensitivity of the assay to desired levels.

Introduction

The LPBE used for detection of antibodies against foot and mouth disease virus (FMDV) is a novel, reliable and reproducible test (Hamblin *et al*., 1986b). It is a prescribed test for international trade and is a useful tool for epizootiological surveys and vaccine potency testing (Hamblin *et al*., 1986a; OIE, 1996). It is based on the traditional virus neutralization (VN) test. In principle, a constant dose of pre-titrated antigen is allowed to react in liquid-phase with serum antibodies. Then free unblocked antigen is assayed by a trapping sandwich ELISA. The antigen dose is selected to give an O.D. value of 1.5; where both the 100% and the 50% OD values lie on the linear portion of virus titration curve (Hamblin *et al*., 1986b), i.e. the amount of antigen will be sensitive to any blocking activity.

One of the components of validation of diagnostic tests for infectious diseases is the determination of assay's performance and characteristics (Crowther, 2001). If the characteristics of the testing system are known, the rate at which false positive (non-specificity) and false negative (insensitivity) can be anticipated for the better judgment of serological surveillance (OIE, 2005).

In this work we present findings that unveil some important determinants of serum blocking activity in the LPBE.

Materials and Methods

ELISA reagents:

All ELISA reagents were prepared by the WRL for FMD, Pirbright, U.K. FMD antigens were type O_1, Manisa batch lot 2590PO31 and type SAT_1 BOT 1/77, batch 14/5/97. Both were supernatants derived from infected cell culture, which have been inactivated with binary ethyleneimine (BEI).

The trapping antibody reagents were rabbit antisera (whole serum) each specific to one type of FMDV; batch 19 to serotype "O" and batch 1 to serotype "SAT_1".

The detecting antibody reagents were guinea pig antisera (whole serum), pre-blocked with non-immune bovine serum (50% v/v). They were of the same specification as the trapping antibody; batch 24 to serotype "O" and batch 2 to serotype "SAT_1".

The anti-species conjugate (Batch 16) was a rabbit anti-guinea-pig immunoglobulin (Ig) conjugated to horse radish peroxidase (HRP) and pre-blocked with non-immune bovine serum (50% v/v).

Control reagents were negative bovine reference serum (Batch 16), serotype O-specific bovine reference antisera; strong positive (Batch 10) and medium positive (Batch 12), and serotype SAT_1-specific bovine reference antisera; strong positive (Batch 1) and medium positive (Batch 4).

Test sera:

A one-hundred serum samples were obtained from apparently healthy cattle above one-year-old, from a slaughterhouse at Khartoum State. Sera were separated by centrifugation and kept at $-20^{o}C$ till use.

Optimum concentrations of ELISA reagents:

The dilutions of ELISA reagents (conjugate, detecting antibody, trapping antibody and antigens) suggested by the WRL were tested in chequerboard titrations. All worked at the expected dilution except the detecting antibody reagents which worked at dilution $^{1}/_{30}$ rather than $^{1}/_{100}$. The Chequerboard titrations were performed as described by Hamblin *et al*. (1986b). The determined optimal concentrations of the guinea pig antiserum and the conjugate were used to determine the optimal concentrations of the trapping antibody for each serotype. Results of antigen titrations are shown in Fig. 1.

Screening assay:

ELISA procedure described by Hamblin *et al*. (1986b) was adopted. Test and control sera were used at the single dilution of 1/16. Test sera were in duplicates and control sera were in quadruples. Antigen control involved four wells

Median antigen OD value for each plate was calculated from the two intermediate values of the antigen replicas. The discarded lowest and highest replica values must fall within the limit of 0.8 to 1.9. Percent inhibition (PI) values of control and test sera were calculated according to the formula:

$$PI = 100 - \left(\frac{\text{Replicate OD of test (control) serum}}{\text{Median OD of control antigen}}\right) \times 100$$

PI values below 50% were considered negative and above 50% positive.

According to WRL for FMD three values of PI for each control should be within limits. These limits for control antigen are between 25 and –25%, for control strong positive serum between 85 and 100%, for control medium positive between 50 and 85% and for control negative serum between 0 and 49%.

Titration assay:

Similar to screening assay except that sera were used in duplicated two fold serial dilution starting from $^1/_{16}$ to $^1/_{128}$.

Results

Performance of the screening test at the recommended antigen dose:

$^1/_{100}$ and 1/10 dilutions of type “O” and "SAT1" antigens, respectively, which gave an OD value of 1.5 and lay on the linear portion of antigen titration curve (Fig. 1), were not sensitive enough to differentiate between normal and medium positive reference sera (Table 1a; b). Both sera, in both assays, showed PI values below 50%. The medium positive serum showed PIs either lower than the normal serum or minus PI values (complete absence of blocking activity). When more type "O" and less type "SAT_1" antigens were used, the blocking activity of the medium positive serum improved over that of the reference normal serum in the first case (Table 2a) and completely disappeared in the second case (Table 2b). i.e antigens at dilution $^1/_{100}$ and 1/10 were not in excess. On the other hand, in each assay half of the tested sera (20 out of 40) showed PI values above 50% (Table 3; 4). Seven sera in type “O” assay and one serum in type "SAT_1" assay had PI values above 90%. This discrepancy, was created by the weak strength of reference positive sera, was thought of as unlikely. It was not limited to type “O” and “SAT_1” reference sera, but was also observed in type “SAT_3” and “A” assays (data not shown). In addition, it was also found to be associated with another phenomenon, e.g. minus PI values in the reference sera as well as fresh test sera. Finally, the increase in antigen concentration had a favourabe influence in PI values and the effect had a certain pattern.

Response to increase of type "O" antigen:

Results presented in Table 5 show that increase in type “O” antigen from $^1/_{100}$ to $^1/_{70}$ increased PI values of different serum groups. Both reference and test sera showed such a response.

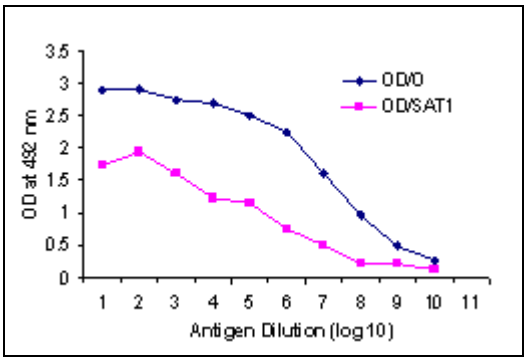


Fig. 1: Titration of FMDV type O and SAT1 antigens

The response was less pronounced in reference positive sera than in their respective counterparts of the test sera. On the other hand, sera of low blocking activity at the antigen dilution of 1/100, others than the strong or medium positive sera, responded to increase in antigen concentration with high rate of increase in PI. In traditional VN test, low concentrations of antigen (virus) rather than high concentrations are expected to be more sensitive in detecting lower amount of antibodies.

Table 1: Performance of screening assay at the recommended antigen dose

Table 1 a: Type "O" (1/100 dilution of antigen):

Materials tested	**1st trial**			**2nd trail**		
	O.D.*	**P.I. range****		**O.D.***	**P.I. range****	
Control antigen	1.399	-2%	19 %	1.542	-23 %	4 %
Reference –ve serum	1.654	-26%	37 %	0.932	10%	43 %
Reference medium +ve serum	1.237	-11%	17 %	1.068	11%	48 %
Reference strong +ve serum	0.243	81%	86%	ND	ND	

Table 1b: Type "SAT1" (1/10 dilution of antigen):

	1st trial	
	O.D.*	**P.I. range****
Control antigen	1.232	-18 % 7 %
Reference –ve serum	1.005	6 % 32 %
Reference medium +ve serum	1.089	-4 % 32 %
Reference strong +ve serum	0.653	43 % 74 %

**=Optical density (average of two medium values out of 4)*

***= Percentage inhibition (lowest and highest values)*

ND =Not done

Table 2a: Effect of increasing type "O" antigen on blocking activity of normal and medium positive reference sera:

Antigen concentration	$^1/_{100}$	$^1/_{90}$	$^1/_{80}$
Control antigen OD values*	1.542	1.560	1.42
Reference –ve serum OD values*	0.932	0.938	0.927
Reference medium +ve serum OD values*	1.068	0.865	0.812

** =Average of two medium values out of 4*

Table 2b: Effect of decreasing type "SAT1" antigen on blocking activity of normal and medium positive reference sera:

Antigen concentration	$^1/_{10}$	$^1/_{11}$	$^1/_{12.5}$
Control antigen OD values	1.232	0.868	0.993
Reference – ve serum PI values	6 13 24 32	-15 -7 9 38	-7 0 10 14
Reference medium +ve serum PI values	-4 1 23 32	-41 -20 -16 -7	-50 -35 -18 33

-ve=negative

Table 3: Performance of type "O" screening assay at different antigen concentrations:

Blocking activity (PI values)	**Distribution of PI values at antigen concentration of**		
	$^1/_{100}$	$^1/_{77}$	$^1/_{70}$
Very high blocking activity (90% or above 90%)	07	13	19
High blocking activity (60% – 89%)	13	05	12
Medium blocking activity 40% - 59%)	5 (all between 40-49%)	12(4 between 40%-49%)	4(1 between 40-49%)
Low blocking activity (less than 40%)	15 (6 -ve values)	10 (2 -ve values)	3 (1 –ve values)

-ve = Negative

Table 4: Performance of type "SAT_1" screening assay at different antigen concentrations

Antigen concentration	$^1/_{10}$	$^1/_{11}$	$^1/_{12.5}$
No. of +ve test sera out of 40 (PI 50-85%)	20	3	4

Table 5: Response to the increase in type "O" antigen concentration:

Serum group	Serum No.	Serum replicates PI values at antigen dilutions of		
		$^1/_{100}$	$^1/_{77}$	$^1/_{70}$
positive group	377	92%. 92 %	92% 94%	97% 97%
	477	N.D.	96% 96%	95% 97%
	Ref.(++)	82%, 83%, 86%	75% 77% 84%	88% 90% 91%
	330	83%, 85%	N.D.	93% 94%
	382	47%, 57%	31% 49%	59% 59%
	383	65%, 71%	38% 51%	79% 81%
Weak Positive Group	Ref.(+)	-11%, 7%, 17%	36 %, 38% 48%	43%, 43%, 56%
	112	3%, 17%	37%, 44%	62% , 64%
	392	-7%, 11%	38%, 57%	67% , 69%
	304	-1%, -25%	43%, 44%	46% , 61%
Normal serum group	Ref.(-)	-33%, -10%, 37%	5%, 10%, 21%	39%, 40% , 49%
	361	-2% , -6%	23%, 25%	22% , 29%
	470	-75%, -85%	23%, 31%	0%, -14%

Pattern of response to the increase of type "O" antigen dose:

Two patterns of response to the increase in antigen concentration were evident (Fig. 2; Table 5); a linear and a curvilinear increase response. Linear increase response was shown by sera that showed high or very low blocking activity at the low antigen dose of 1/100. These comprised strong positive test sera, medium positive reference antiserum and similarly weak positive test sera. Sera that showed medium blocking activity at the low antigen dose of 1/100 showed the curvilinear increase response. These include the strong positive reference antiserum and medium positive test sera. Normal reference serum and normal test sera that showed low blocking activity at the low antigen dose showed also an increase in blocking activity that was unsteady as well as limited to below 50% PIs. Seven out of 8 normal test sera which were detected during this work showed PI values below 40% at antigen dilution of $^1/_{70.}$

Reflection of the described patterns is quite apparent in comparing performance of type "O" screening assay at different antigen concentrations (Table 3). With antigen concentration, numbers of sera with high (above 90%) and low (below 40%) blocking activities responded by linear increase in the former and linear decrease in the latter. Detected blocking activity between 60% and 89% followed exactly the described pattern for medium positive test sera.

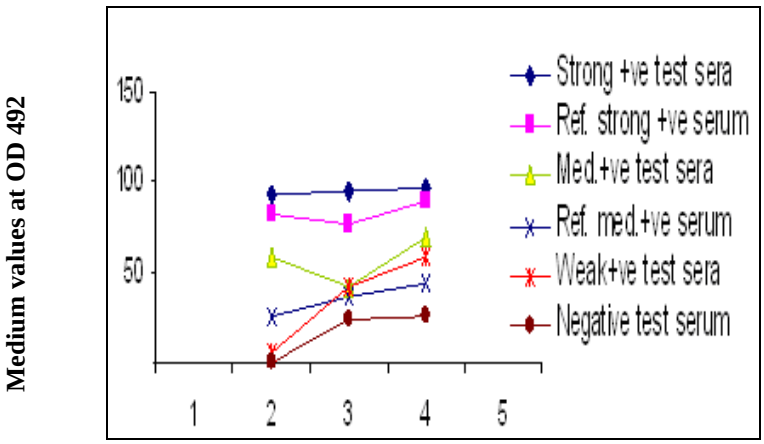


Fig. 2: Blocking activity of test sera in relation to reference sera at different Type O antigen concentrations.

Minus PI values:

One of the main findings in this work was the minus values of PI. It was observed in reference as well as test sera. Its association with low concentration of antigen is evident (Table 2b; 3; 4). Table 2b shows that decreased antigen concentration has unequivocally led to complete absence of blocking activity of weak positive sera (also medium positive reference serum) rather than normal sera. In type "O" screening assay, six test sera had minus PI values at low antigen doses, four of them changed to PI values above 50% (positive) at the highest antigen dose (Table 3).

Performance of type "SAT$_1$" screening assay at different antigen doses:

The general features displayed by type "O" screening assay are all shown in "SAT$_1$" screening assay (Tables 1b; 2b; 4).

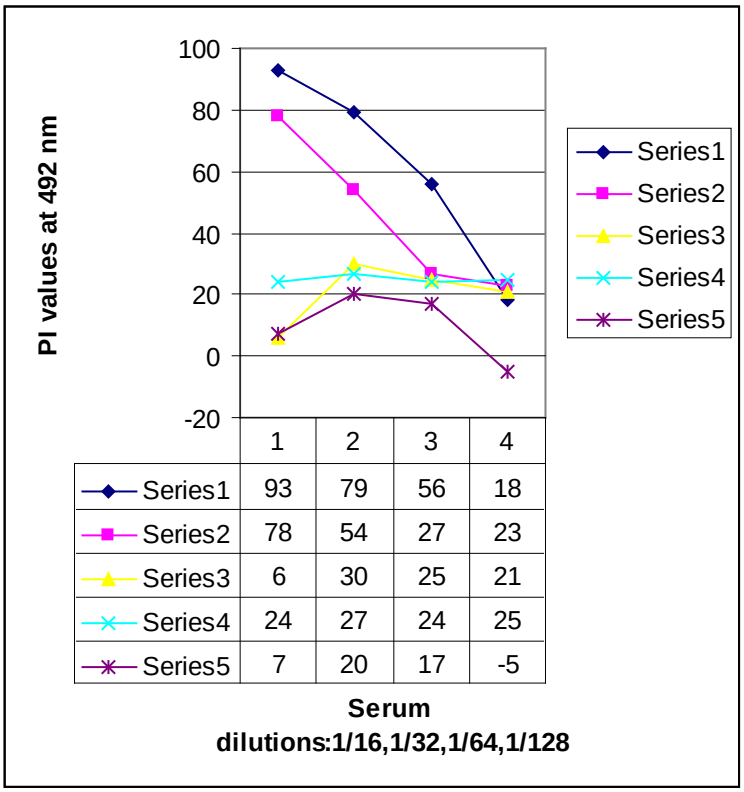
Type "O" titration assay:

The important finding in this work was that the increase in antigen concentration raised the blocking activity and turned some negative sera positive. To show that this change was not arbitrary, two of these sera (series 3 and 4) were titrated using 1/77 dilution of type "O" antigen. Together with them three sera that showed positive (series 1 and 2) or negative (series 5) results, were all titrated using the same antigen concentrations throughout. Results are presented in Fig. 3. It is evident that these sera, unlike the positive sera, showed no titration at all, and unlike the normal serum they showed no end points. Their PI

values through the successive serum dilutions, either remained the same or even showed a rise.

Discussion

In essence, the data presented in this work showed that increased antigen concentration in screening assays increases PI, while decreased antigen concentration decreases PI. This might be suggestive of an inhibitory effect on blocking activity due to serum viscosity when sera were used at dilution $^{1}/_{16}$. Clue for this suggestion



	1	2	3	4
Series1	93	79	56	18
Series2	78	54	27	23
Series3	6	30	25	21
Series4	24	27	24	25
Series5	7	20	17	-5

Fig. 3: Type "O" titration assays (antigen dil.= 1/77)

was also observed in titration assay. In titration assay (Fig.3), some sera showed higher blocking activity at the serum dilution of $^{1}/_{32}$ rather than at $^{1}/_{16}$. On the contrary, however, increased antigen concentration in screening assay decreased the blocking activity of type “O” strong positive reference serum and medium positive test sera before raising it again (Table 5; Fig. 2). Moreover, while the blocking activity of these sera was decreasing, that of strong positive test sera was increasing (Fig. 2).

Abu Elzein *et al*. (1987) using type-specific indirect ELISA for detection of antibodies against FMDV, found it necessary in spot test to use sera at dilution $^{1}/_{100}$ or $^{1}/_{200}$. At lower dilution, non-specific serum protein binding took place and interfered with results of the test. Here, weak positive sera used at dilution $^{1}/_{16}$, including medium positive reference sera (Table 1a; b), showed PI values lower than those of normal sera or a complete absence of blocking activity (minus PI values). Since the factor of serum viscosity in the liquid phase has been excluded, another possibility of serum protein binding in the immune plate is raised. Because the interference with results of the test is mostly associated with the weak positive serum group rather than with other groups, therefore, serum protein binding, if occurred, is expected to be most pronounced in case of weak positive sera.

On the other hand, since increased antigen concentration did not simultaneously increase PI values in all serum groups. the observed increase of PIs followed certain pattern (Fig. 2), and because in both types of assay, "O" and "SAT_1", positive test sera were detected while medium positive reference antisera showed blocking activity below 50%, it is justifiable to conclude that the detected blocking activity is not related to the amount of antigen but to the relative concentration of antigen and antibody and to the nature of antibody (avidity).

During the liquid phase of the test, antibodies in positive sera react with antigen to form immune complexes, which consequently reduce the amount of free antigen available for trapping in the immune plate. Generally speaking, many factors are known to affect formation and fate of immune complexes. One of these factors, and the most important, is the ratio of antibodies to antigen (Hugh Fudenberg *et al., 1978*). When antigen or antibodies are in excess the formed immune complexes are smaller in size than when antigen and antibodies are about equal. Small complexes have few epitopes while large complexes have numerous ones (Crowther, 2001). It could be expected that the few epitopes on small complexes are more efficiently blocked than the numerous epitopes on large complexes. Presence of infectious immune complexes (not efficiently neutralized) which interferes with neutralization of FMDV in cell culture has been established (Booth *et al*., 1978). Accordingly, in the system of LPBE, some of the numerous epitopes on the large complexes would not be covered completely by antibodies, remain available for binding by the immobilized antibodies in the immune plate, raise OD and decrease PI. On the other hand, excess antibody and excess antigen immune complexes would be more efficiently blocked and result in high PIs.

Observable effect of equivalent antigen antibodies ratio and large immune complexes (high OD and low PIs) is mostly to be expected when sera are weakly positive and antigen is relatively low. In this work most pronounced interference with the test results was observed in weak positive sera at a comparatively low antigen concentration of $^{1}/_{100}$ (type "O" assay) or ^{1}

On the other hand, observed differences between reference and test sera could be explained by the factor of serum avidity. Reference antisera are products of experimental infection and positive test sera are sero-converts following natural exposure. Antibodies that follow natural infection have higher affinity than those which follow experimental infection (longer course of natural infection). High affinity antibody only requires low amount of antigen concentration to achieve binding of antigen to the half of its binding sites (Hugh Fudenberg *et al.,* 1978). Moreover, low affinity antibody (experimental infection) would produce low affinity immune complexes which, like large immune complexes, might interfere with the ELISA system, in the aforementioned described manner, lowering the detected blocking activity. According to Hamblin *et al*. (1987), difference in the

antibody response following natural and artificial challenges is distinguishable by the LPBE and is even more apparent than in neutralization test.

Whereas the reaction specificity at high antigen doses is guaranteed by the improvement of blocking activity of reference sera, it also gains support from results of titration assays (Fig. 3). According to Crowther (2001) when normal sera are titrated they either show percentages of inhibition lower than the plate background or (minus PI values) (Table 2a, b). On the other hand, this is quite consequent with the observation that the only non-specific among the tested (Minus PI values) showed the end point of titration. False negative (Fig. 3: series 3 and 4) sera showed no end points (Fig. 3) or end points with very low mean PIs (data not shown). Moreover, sera exchanged to positive, in higher antigen concentrations. At the lower antigen concentration of 1/100, strong positive sera were heterogeneous antibody population, avidity of a serum may change on dilution (Crowther, 2001) and result in similar PIs.

1/12.5 (type "SAT1" assay), e.g. normal positive reference (and weak positive test sera) either showed end points which were close to the plate background (0% in this case) (Serum 470 (Fig. 3, series 5), which was diagnosed as negative in this work as the non-specific component reacted (Group III sera) is associated with the reaction rather than with normal sera.

The observed pattern of response that followed increased antigen concentration (Fig. 2) could be explained on the basis of higher antibody ratio. At the higher antigen concentration, the PIs were similar to serum dilutions. In excess of antibody with increased antigen concentration, since they contained high amount of antibodies, they remained at antibody excess, and showed limited linear increase in PIs. Riskalla et al. (1995) reported some discrepancy in detection of weak positive sera by the LPBE. This to some extent, resembles the difficulty of detecting weak positive sera encountered in this work. This work demonstrates how antigen/antibody ratio and serum avidity may shape the antigen dose response in the LPBE. Increased antigen dose, within the limits of the test, raises sensitivity and improves specificity, of weak positive sera, at the lowest antigen concentration of 1/100, were more efficiently blocked and high affinity antibody in sera with weak avidity would react more preferentially to the area of antigen).

Most positive sera were at antibody excess, but since they contained comparatively low amounts of antibodies, they moved with increased antigen concentration to the equivalent antigen/antibody ratio and showed decrease in PIs. By further increasing antigen doses to the area of antigen excess and showed a rise in PIs. Thus the result, response would be curvilinear. Alternatively, the screening at the low antigen concentration, complexes are more formed antigen/antibody and high affinity attained the lowest amount of antibodies. With increase of antigen concentration they moved to the area of antigen excess and showed a linear increase in PIs.

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