*The Sudan J. Vet. Res. (2007), **22:** 25-36*
With 2 tables and 4 figures in the text.

Selection of Antigen Concentration for Foot and Mouth Disease Virus Antibody Detection in Liquid-Phase Blocking ELISA (LPBE) Spot Test

A/ Raouf, Y.;[1] Ali, B. H[2].; El Amin, M. A[1].; Habiela, M[1].; Salih, M. M[1].; Mahasin E. A/ Rahman[1] and Nur Eldin, H[1].

(1) Central Veterinary Research Laboratories Centre, P.O.Box 8067 (Al Amarat), Khartoum, Sudan. (2) Faculty of Veterinary Medicine, Sudan Univerisity of Science and Technology, P.O. Box 204, Khartoum North. Sudan.

ملخص البحث

لتحديد الجرعة الاكثر ملائمة من المستضد لاختبار التلازن المناعي المتصل بالإنزيم ذى الطور المائع المحصر (Liquid-Phase Blocking ELISA) لمقايسة المسح (Screening Assay) لمرض الحمي القلاعيه' تمت معايرة عينات مصلية للأضداد النوعية للمستضد "SAT1" باستعمال جرعات مختلفة من المستضد المذكور بدءا" بالجرعة التي تعطي معدل كثافة ضوئية(OD value) بمقدار1.5. وجد أن الجرعه الاكثر ملائمه تحدث منحنيات معايرة مماثله لمختلف مجموعات المصل الموجبه (عالية–متوسطة–ضعيفة).

عند استعمال تركيز مناسب من المستضد يكون من الممكن تسجيل أعلي نشاط إحصاري (Maximum plateau height of blocking activity) (أقرب ما يكون الي 100%) كما أن العلاقة التلازمية (correlation) بين تخفيفات الأضداد النوعية في العينة المصلية و قلة النشاط الأحصارى تكون في أحسن حالاتها.

عند استعمال تركيز أقل يكون أعلي نشاط إحصاري يمكن تسجيله أقل مما يجب عليه أن يكون (أقرب إلي% 70 و 80%) كما أن النشاط الأحصاري المسجل ينخفض بمعدلات مرتفعة لا تتناسب و تخفيفات الأضداد النوعية في العينة المصلية . في هذه الحالة يمكن أن تكون المعايرة اقل حساسية(less sensitive) و أيضا" ذات نوعيه أقل (less- specific).

عند استعمال تراكيز عالية من المستضد يكون النشاط الإحصاري أقل، خاصة مع العينات المصلية ذات التركيز المنخفض و المتوسط من الأضداد النوعية.

Summary

To determine the optimal antigen dose for anti-type SAT1 virus antibody detection liquid-phase blocking ELISA screening assay of FMD, a panel of local and control sera were titrated using different concentrations of the antigen, starting from a concentration that gives an OD value of 1.5.The optimal antigen concentration was found to produce parrallel titration curves for the different positive serum groups (strong, medium and weak). At such concentration, the screening assay correlates well with the titration assay. The sera that showed the highest positive percentage inhibition (PI) value in screening assay showed the highest titre and vice versa. Maximum plateau height of blocking activity (near 100%) was produced and good correlation between serum dilutions and the decrease in positive blocking activity did frequently exist.

At low antigen concentrations, no correlation was observed between screening and titration assay; sera that showed similar positive PIs in screening assay showed different titres or vice versa. Comparativley low plateau height (70% and 80%), poor correlation and/or sharp decrease in detected positive blocking activity following serum dilution, marked these lower concentration. The assay might be both insensitive and less specific. High antigen doses were marked by a decrease in the detected blocking activity, particularly, of weak and medium positive sera (insensitivity).

Introduction

The liquid-phase blocking ELISA (LPBE) used for foot-and-mouth disease (FMD) antibody detection is a novel and useful test (Hamblin *et al*, 1986a). It is intended for serological surveys (Hamblin *et al*, 1986b). In these surveys large numbers of sera are tested, usually, at a single dilution (spot test) to economize efforts and reagents. Nevertheless, spot tests (screening assays) should be sensitive, specific and reliable.

In the LPBE spot test, sera are tested at a single dilution of 1/16 and the antigen concentration for the test is selected to give an OD value between 1 and 1.5 (LPBE kit for FMD serology information, Pirbright Laboratory, UK) or 1.5 (Hamblin *et al*, 1986a). According to Crowther (2001a) for reliable results in spot tests, serum dilutions to be employed should be selected from serum titration curves where the latter are almost parallel. Abu El Zein *et al* (1987) screened sera for antibodies against FMD virus by indirect ELISA at dilutions 1/100 and 1/200 to avoid non-specific serum protein binding that takes place at lower serum dilutions and causes a rise in OD values. Raouf *et al* (2006) studied the antigen concentration response in the LPBE and showed that antigen/antibody ratio is an important determinant of serum blocking activity. They have shown that most reliable results of the spot test are obtained when antigen/antibody ratio is at excess of antigen. This work is intended to show that the antigen optimum concentration for the spot test would produce ideal parallel serum titration curves as those described by Crowther (2001a). Only then, testing of sera at dilution 1/16 where positivity is equivalent to a titre of 1/45 ($10^{1.65}$), which is the serum positive threshold as determined by serum neutralization test, would be sensitive, specific and reliable.

Materials and Methods

ELISA reagents:

SAT1 LPBE reagents used in the study throughout, were obtained from the World Reference Laboratory for FMD (WRL), Pirbright, UK.

Test sera:

Reference strong and medium positive bovine antisera (C++ and C+) and local breeds' sera were used throughout the study. Local breeds' sera were obtained from apparently healthy cattle above one-year-old. They were collected in plain vaccutainers, separated by centrifugation and kept at -20^{o}C till used.

Optimum concentrations of ELISA reagents:

ELISA reagents were specified in details by the World Reference Laboratory for FMD, Pirbright ,UK.

Screening and titration assays:

Generally, ELISA procedure described by Hamblin *et al* (1986a) was adopted. Method in detail was described elsewhere(Raouf *et al*,2006) .

In screening assay, sera were tested at a single serum dilution of 1/16 and results were expressed as percentage inhibition (PI) values (average value of two replicas). The positive threshold is at 50% PI value.

In titration assay, test sera were examined at two fold serial dilution ranging from 1/16 to 1/128 resulting in a final serum dilution of 1/32 to 1/256 after addition of an equl volume of antigen. The titre of a test serum demonstrating PI values above 50% was assessed according to the table 1 which was supplied with the ELISA reagents.

Controls for each set of test (screening or titration) included antigen, normal reference serum, medium and strong positive reference antisera.

Table I: Test serum antibody titre

Test serum dilution	**Sera with different replicates of PI values>50 (+)**							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1/32	+ -	+ +	+ +	+ +	+ +	+ +	+ +	+ +
1/64	- -	- -	+ -	+ +	+ +	+ +	+ +	+ +
1/128	- -	- -	- -	- -	+ -	+ +	+ +	+ +
1/256	- -	- -	- -	- -	- -	- -	+ -	+ +
Antibody titre	32	45	64	90	128	181	256	>256

Results

Fig. 1 shows that 1.5 OD value was produced by about 1/20 dilution. Twice this dilution (1/10), which was equivalent to 600 µl of antigen in 6 ml, was used in the starting assay. Antigen dose was increased (Table 2) to 650,725, 750 and 800 ul in 6 ml, ie from 1/10 to 1/7.5 dilution, respectively.

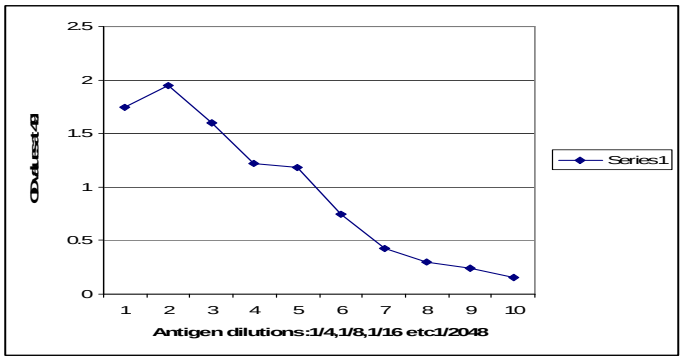


Fig. (1): Antigen titration curve.

Table 2: Average PI values and titres at different antigen doses

Ag dose /6ml	**600** µl	**650** µl		**725** µl		**750** µl		**800** µl
OD value	1.232	1.273		1.210		1.565,1.279,1.354 1.95		2.724
Serum No.	Spot t. (PI)	Tit. (titre)	Spot test (PI)	Tit. (titre)	Spot test (PI)	Tit. (titre)	Spot test (PI)	Spot test (PI)
C++	47%	-ve	12%	-ve	31%	128	83%	77%
C+	26%	N.D.	N.D.	-ve	6%	N.D.	28%	7%
1090	88%	181	85%	90	84%	90	87%	82%
539	37%	-ve	28%	-ve	33%	90	73%	60%
1753	76%	N.D.	N.D.	90	77%	-ve	41%	39%
1744	35%	-ve	20%	-ve	15%	64	60%	50%
1008	17%	-ve	21%	-ve	5%	-ve	33%	27%
375 b	N.D.	64	85%	90	85%	181	94%	93%
484 kh	N.D.	-ve	25%	N.D.	N.D.	N.D.	60%	N.D
206 kh	N.D.	45	87%	N.D.	N.D.	N.D.	92%	N.D
207 kh	N.D.	90	86%	N.D.	N.D.	N.D.	82%	N.D
261 kh	N.D.	Reversed curve	50%	N.D.	N.D.	N.D.	60%	N.D

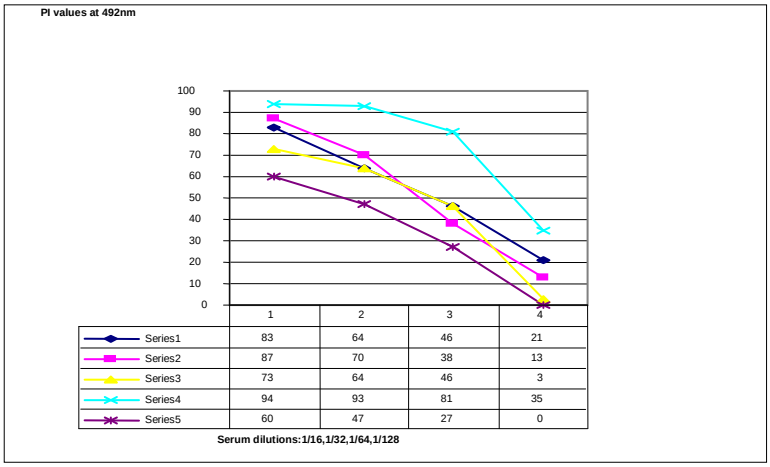
Ag*: antigen; OD: average optical density value; Spot t.: spot test*;
Tit.*.: titration assay; PI: percentage inhibition average of two replicate values at dilution 1/16 of serum;. -ve: negative.*

Results presented in Fig. 2a show that titration curves of positive sera at the antigen dose of 750 µl /6 ml (the optimum dose) were parallel. This parallelism reflects the presence of only maximum plateau height (above 90% PI values) and good correlation between serum dilutions and detected blocking activity for each serum, ie good titration. Accordingly, the

serum that showed the highest PI value at dilution 1/16 showed the highest titre and vice versa (Table 2).

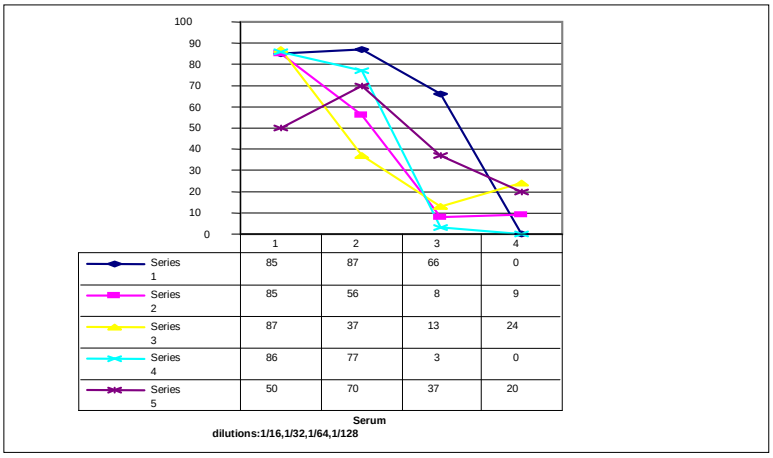
Fig.2b and Fig.2c show titration curves of positive sera at antigen doses of 650 µl/6ml and 725µl/6ml, respectively. In Fig. 2b, poor correlation between serum dilutions and detected blocking activity was observable to the extent that in one case, blocking activity increased with serum dilution from 50% to 70% (serum no. 261 kh). The lack of parallelism was evident in that four sera at dilution of 1/16, showed PI values between 85 and 87 yet showed titres that ranged from 181 to 45 (Table 2). The fifth serum (serum no. 261kh), was graphically untiterable since it had two positive end points; one at dilution 1/16 and the other between dilution 1/32 and 1/64 (Fig. 2b). By increasing antigen dose to 725ul/6ml some improvement was observed. Detected positive sera at this antigen concentration showed at dilution 1/16, PI values between 77 and 85 and all produced a titre of 90 (Table 2). Yet, graphically (Fig. 2c), showed poor correlation between serum dilution and blocking activity. Plateaues of low height were observable (84-81 and 77-76). Sera no.1090 and no.1753 showed sharp decline in PI values, following the 2^{nd} and 3^{rd} serum dilution, from 32 to -49 and from 76 to 9, respectively, whereas, serum no.375b showed no end point.

Increase in antigen dose to 750 µl/6 ml was also associated with increase in sensitivity of the test. Four sera (strong positive reference antiserum, 539, 1744 and 484 KH), out of the twelve examined, that showed negative result when tested 10 times at low antigen doses turned positive at the antigen dose of 750 µl /6 ml (Table 2). Moreover, all sera that scored positive whenever tested (five sera), showed the highest PI values for the 1/16 serum dilution at this antigen dose except serum No.207 kh. Increase in antigen dose over 750 µl /6 ml to 800 µl /6 ml resulted in decrease of PI values of positive sera at dilution 1/16 though they remained positive (Table 2). The observed increase of sensitivity at the optimum antigen concentration of 750 µl /6 ml was not arbitary. Sera that changed to positive or showed marked increase in titre at this antigen concentration displayed similar charcreristics in the shape of the titration curve at lower antigen doses. The titration curves of these sera at low antigen doses (Fig. 3) showed no end point; unlike their titration curves at the optimum antigen concentration (Fig. 2a).



Series=strong +ve referenc antiserum; series 2=serum No. 1090; series 3=serum No. 539; series 4= serum No. 375(b); series 5= serum No. 1744.

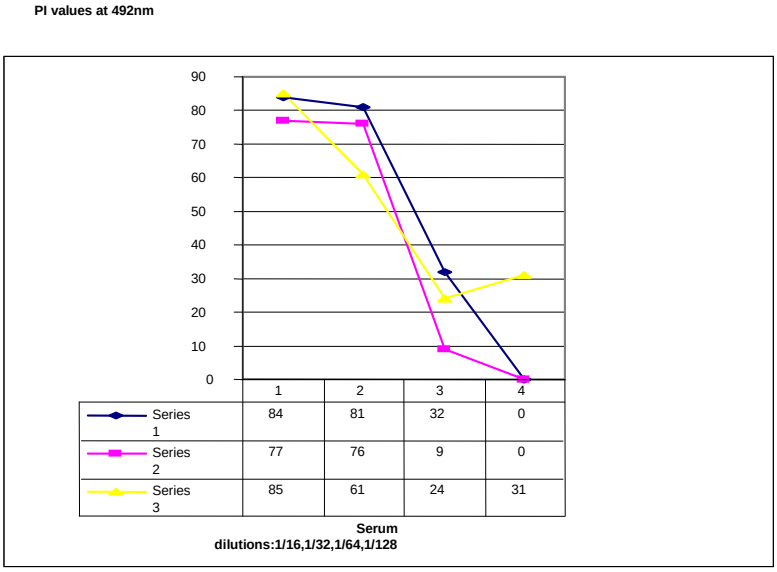
Fig. 2a: Titration curves of positive sera at antigen concentration 750µl / 6ml.



N.B. 0= -ve value

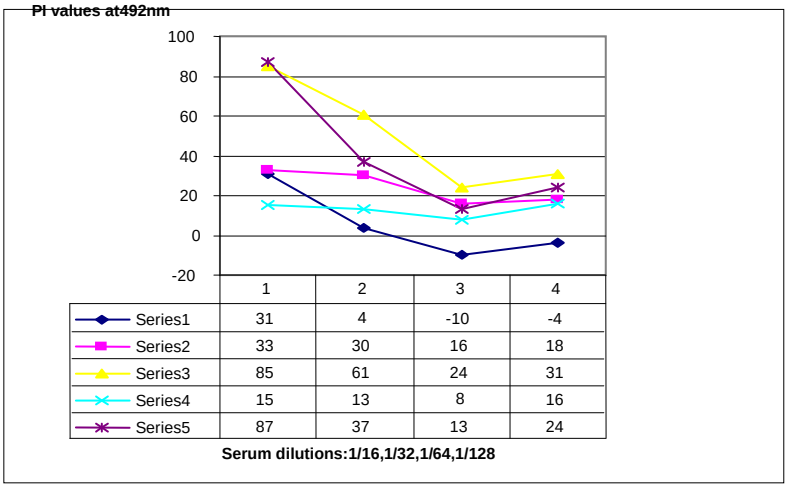
Series1= serum No 1090 ; Series 2= serum No. 375(b).; Series 3= serum No. 206kh.; Series 4= serum No. 207kh.; Series 5= serum No. 261kh.

Fig. 2b: Titration curves of positive sera at antigen concentration 650 µl /6 ml.



N.B: 0= -ve value
Series1= Serum No 1090.; Series 2= Serum No. 1753.; Series 3= Serum No. 375(b).

Fig. 2C: Titration curves of positive sera at antigen concentration 725 µl /6 ml



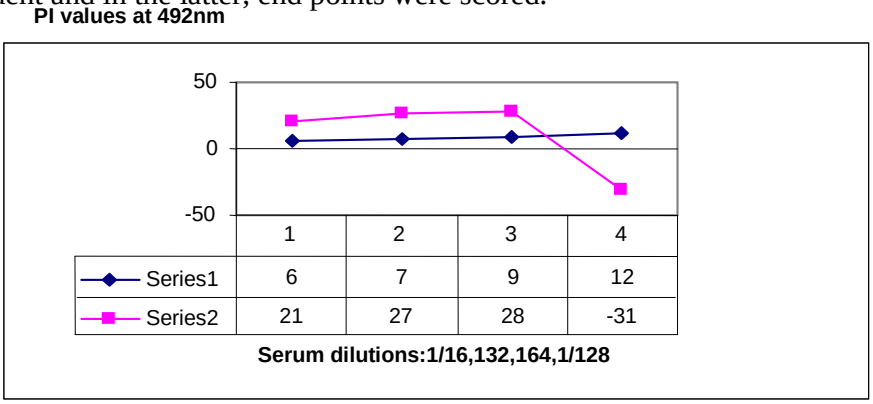
Series: 1 = Strong +ve reference antiserum.; Series 2 = Serum No. 539.; Series 3 = Serum No. 375(b).; Series = Serum No. 1744.; Series 5 = Serum No. 206kh

Fig. 3: Insensitivity at low antigen concentration (characteristics of serum titration curves)

Change in specificity was also observed at the optimum antigen dose (750µl/6 ml). Serum no.1753 had turned negative at the optimum antigen concentration and another serum (no. 1090) showed a marked decrease in titre from 181 at low antigen dose to 90 at the optimum concentration (Table 2).

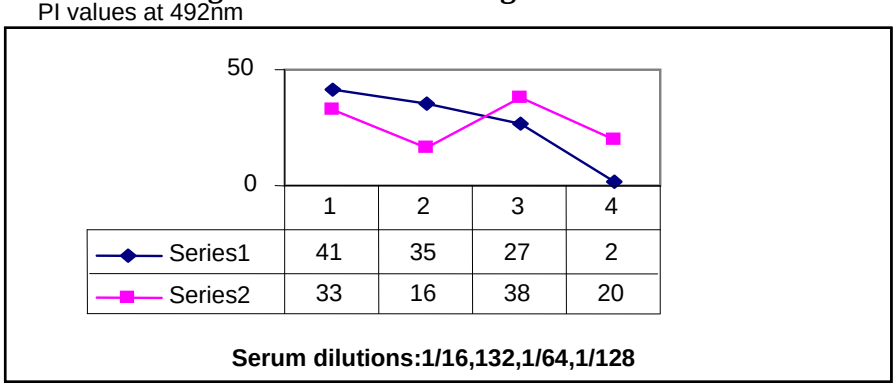
Similar to the case of sensitivity increased, the shape of the titration curves of these sera at low antigen concentration displayed similar charcteristics (Fig.2b;c). They showed low plateau height and sharp decline in PI values at one point or another in their titration curves. These characters disappeared at the optimum antigen concentration (Fig.2a). The change is judged as an increase in specificity since the low plateau height and the sharp decline in PI values could be significant of low avidity or low quantity of antibodies.

Titration of negative sera at low and optimum concentration is shown in Fig. 4a and 4b, respectively. In the former, reversion of the titration curves was evident and in the latter, end points were scored.



Series1=Medium positive reference antiserum.; Series 2= Serum NO. 1008.

Fig. 4 a: Titration of negative sera at low antigen concentration.



Series 1= *serum No. 1753; series 2=serum No. 1008*

Fig. 4b: Titration of negative sera at the optimum antigen concentration.

Discussion

Marked differences in detected serum positivity, titres and titration curve characters were observed using different dilutions of antigen. According to kit information, antigen dilution that gives an average OD value above 1.0 should be selected and antigen OD value limits are defined as 0.8 and 1.9. In this respect, all the 1st four antigen concentrations, 600,650,725 and 750 µl /6 ml, produced OD values within these limits. However, reproducibility of results was obtained when the antigen concentration of 750 µl /6 ml was used while OD value of antigen varied from 1.2 to 1.9.Moreover , increase in antigen concentration above 750 µl to 800 µl /6ml, with associated OD value of 2.7, did not do much change in results (strong, and medium positive and negative sera). These results indicate clearly the significance of selecting a optimal antigen dose for the screening assay. The response to the increase in antigen dose by ideal serum titration curves is an additional evidence for the essential role played by factors that govern antigen-antibody reactions in determining the outcome of the assay (Raouf *et al*, 2006).

The present results show unequivocally that only when serum titration curves are parallel the screening assay (result of testing sera at the single dilution of 1/16) correlates well with the titration assay. Crowther (2001a) pointed out that where serum titration curves are parallel, any point on them can be taken for comparison of samples. In this work, the result was achieved by increased antigen concentration. This is advantageous in avoiding the use of a serum dilution other than 1/16, which is the serum positive threshold as determined by serum neutralization test (the golden standard). Moreover, increased antigen concentration eliminates the low plataeue heights (sera no. 1090 and 1753). Low plateaue heights, according to Crowther (2001a) are uncommon when using polyclonal antibodies, but common when using monoclonal antibodies (mAbs). The optimum antigen concentration produced one plateaue height of PI value above 90% and near to the test limit of 100% blocking activity (serum no. 375b). Not that merely, the optimum antigen concentration produces also end points in the titration curves of normal sera (Fig.4b). According to Crowther (20001b), in an optimum screening assay, normal sera should show end points. This is shown only by one serum out of the two investigated at the low antigen doses (Fig.4a).

When dealing with screening assay, of equal significance to the production of parallel serum titration curves is the analysis of these curves (Crowther, 2001a). According to growther (2001 b) serum avidity changes

with serum dilution which may be responsible for poor or good correlation between serum dilution and detected activity. In this respect, sera that showed relatively good avidity (375 b, 206 kh and strong poitive reference antisera) or relatively poor avidity (539 and 1744), as indicated by the correlation between serum dilution and detected blocking activity at low antigen doses (Fig. 3), all changed to positive or showed high PI values at 1/16 dilution when tested with the optimum antigen concentration (Table 2). The explanation is that, sera of mixed population, when the quantity of high avidity antibody is relatively high, will react more perferntially at high serum or high antigen concentration and vice versa. On the other hand, a medium positive reference antisrum and serum no. 1008 that showed the poorest avidity as indicated by complete reversion of the titration curve at low antigen concentrations (Fig.4a) remained negative at the optimum antigen concentrations (Table 2). The observed increased sensitivity following the increase in antigen concentration of the test agrees with our previous findings (Raouf *et al.*, 2006). In that work, it was concluded that low affinity immune complexes particularly at low antigen oncentration would decrease detected blocking activity. Low affinity immune complexes are produced by sera of mixed population when low avidity antibody react more perferntially. This analysis, the agreement with our previous findings, and the observed similarity of titration curves in Fig. 3, all taken together, clearly demonstrate the specificity of the observed changes.

Nevertheless, strictly speaking, low specificity is associated with false positivity. Serum no. 1753 was poitive at low antigen doses and turned to negative at the optimum antigen concentration. However, this serum in particular had showed the lowest detectable plateau height. Moreover, it showed a very sharp decline in PI value from 76% to 9%. Sera that showed a similar sharp decline (Fig 2 a;b) when tested with the optimum concentration, showed either lower titre (serum no. 1090) or lower PI values at dilution 1/16 (serum no. 207 kh). According to Raouf *et al* (2006), at a given concentration of antigen, antibody/antigen ratios are at excess of antibody, equal ratio or excess of antigen. By the increase in antigen concentration, they either remain at antibody excess showing similar high PIs (serum no. 375b), or move from antibody excess and equal ratio to antigen excess showing lower (equal ratio region) then higher PIs and turn to positive (strong positive reference antisera, sera no. 1744 and 539). At the low antigen concentration, if a serum is already at antigen excess, which imples its low content of antibodies, then by further increase in antigen dose it may show low PIs and turn to negative. This my explain the negative

response of serum no. 1753 in comparison with the aforementioned sera that turned to positive at the optimum antigen concentration. The low antibody content of this serum could also be deduced from the low plateau height and the sharp decline of its titration curve. This analysis ascertain the high specificity of the reaction at the optimum concentration in comparison with low doses. Positivity at concentrated antigen is expected to be more specific than at diluted one. In typing of FMD virus by serum neutralization test, FMD virus is used undiluted to avoid cross reactivity (Rweyemamu *et al.*, 1978). Perhaps such a positive reaction as described for serum no. 1753 at low antigen concentration is responsible for the described relatively low specificity of the LPBE by the OIE (2005). Nonetheless, Crowther (2001a) pointed out that, a balance should be set between sensitivity and specificity for selection of a proper serum dilution for the spot test. In this work, it is evident that sensitivity is high at optimum antigen concentration. Sensitivity begin to decrease when antigen is inceased above 750 ul in 6 ml as evidenced by decrease of all PI values of positive sera.

It is hoped that selection of an optimum antigen dose for the assay would increase its specificity, sensitivity and reliability. On-going efforts aimed at increasing the keeping quality of the antigens used in the assay would still add further dimmension to its usefullness.

Acknowledgements

Thanks are due to the staff member of the Department of Foot and Mouth Disease for their technical assistance. We wish to thank the Director of the Central Veterinary Research Laboratories and the Director General of the Animal Resources Research Corporation for their permission to publish this work.

References

Abu El Zein, E. M. E.; Newman, B. J.; Crowther, J. R.; Barnett, I. T. R. and McGrane, J. J. (1987). *Rev. Elev. Med. Vet. Pays Trop.,* **40** (1): :7-12.

A/ Raouf, Y. A.; El Amin, M. A.; Salih, M. M.; Mahasin E. A/ElRahman; Ali, B.H.; El-Eragi, A. M. S.; Mahgoub, M. I.; Habila, M. and Nur Eldin, H. (2006). *Sudan J. Vet. Res.,* .21: 25-37.

Crowther, J. R. (2001a). Use of indirect ELISA to titrate antibodies. In: *The ELISA Guidebook. 1st edn. Human press Inc., Totowa, New Jersey, USA.* Pp. 170-181.

Crowther, J. R. (2001b). Theoretical consideration, in *"The ELISA Guidebook", 1st edn. Human press Inc., Totowa, New Jersey, USA.* Pp. 115-152.

Hamblin, C.; Barnett, I.T.R. and Hedger, R.S. (1986a). *J. Immunol. Methods*, **93:** 115-121.

Hamblin, C.; Barnett, I.T.R. and Crowther, J. R. (1986b). *J. Immunol. Methods*, **93**: 123-129.

OIE. (2005). Terrestrial Animal Health Standards Commission**.** *Guidelines for the surveillance of Foot and Mouth Disease.* Appendix VIII, Article 3.8.7.6.

Rweyemamu, M. M; Booth, J. C.; Morwen, H. and Pay, T. W. F. (1978). *J. Hyg. Camb.*, **81**, 107-123.