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A systematic review and meta-analysis of enzyme-linked immunosorbent spot (ELISPOT) assay for BK polyomavirus immune response monitoring after kidney transplantation

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ABSTRACT

BK virus (BKV) infection after kidney transplantation can cause BKV nephropathy (BKVAN) resulting in graft dysfunction and allograft loss. The treatment for BKVAN is reduction of the immunosuppressive load which increases the risk of kidney transplant rejection. There is no biomarker to monitor BKV activity besides BK viral load. The value of the Enzyme-Linked Immunosorbent Spot (ELISPOT) assay as a tool to monitor the recipient's anti-BKV immune response after transplantation was investigated systematically. Electronic databases, including MEDLINE, Scopus, and the Cochrane Central Register of Controlled Trials were searched for studies of ELISPOT evaluating the immune response against BKV. BKV status was categorized as "active BKV infection" and as "resolving BKV infection". Random-effects model meta-analysis was performed to determine the diagnostic performance of the ELISPOT assay, after stratifying patients into groups based on positive and negative ELISPOT results. One-hundred twenty-seven articles were identified of which nine were included. Patients with negative ELISPOT had an increased risk of having active BKV replication (odds ratio of 71.9 (95%-CI 31.0–167.1). Pooled sensitivity was 0.95 (95%-CI 0.89–0.98) and specificity was 0.88 (95%-CI 0.78–0.94). The standardized mean difference of the number of IFN-γ producing cells between patients with active BKV infection compared with patients who had resolving BKV infection was -2.09 (95%-CI -2.50, -1.68). The ELISPOT assay is a useful tool for BKV risk assessment and in combination with BKV load may support clinicians in guiding immunosuppressive therapy in patients with BKV replication.

1. Introduction

Patient and kidney transplant survival have much improved compared with the previous era due to better immunosuppressive drug therapy and a significant increase in the knowledge of transplantation immunology [1-3]. However, infectious complications, particularly BK virus (BKV) infection, is an important obstacle to allograft longevity [4-8].

BKV replication is the consequence of both the reactivation, which is more common, or *a primo* infection [9]. After kidney transplantation, 30–40% of patients develop early BKV replication which manifests as BK

viruria, whereas 10–20% will progress to BK viremia if the immune response fails to contain BKV replication. Eventually, BKV-associated nephropathy (BKVAN) occurs in 1–10% of kidney transplant recipients [10]. The incidence of BKVAN is higher among patients who receive more potent immunosuppression, which is more common these days as more patients with higher immunological risk receive transplants [7, 11-13]. Currently, there is no established treatment for BKVAN [5,7,8, 14]. The general recommendation is to lower the patient's net immunosuppressive state[15], however, this may increase the donor-specific immune response and result in rejection.

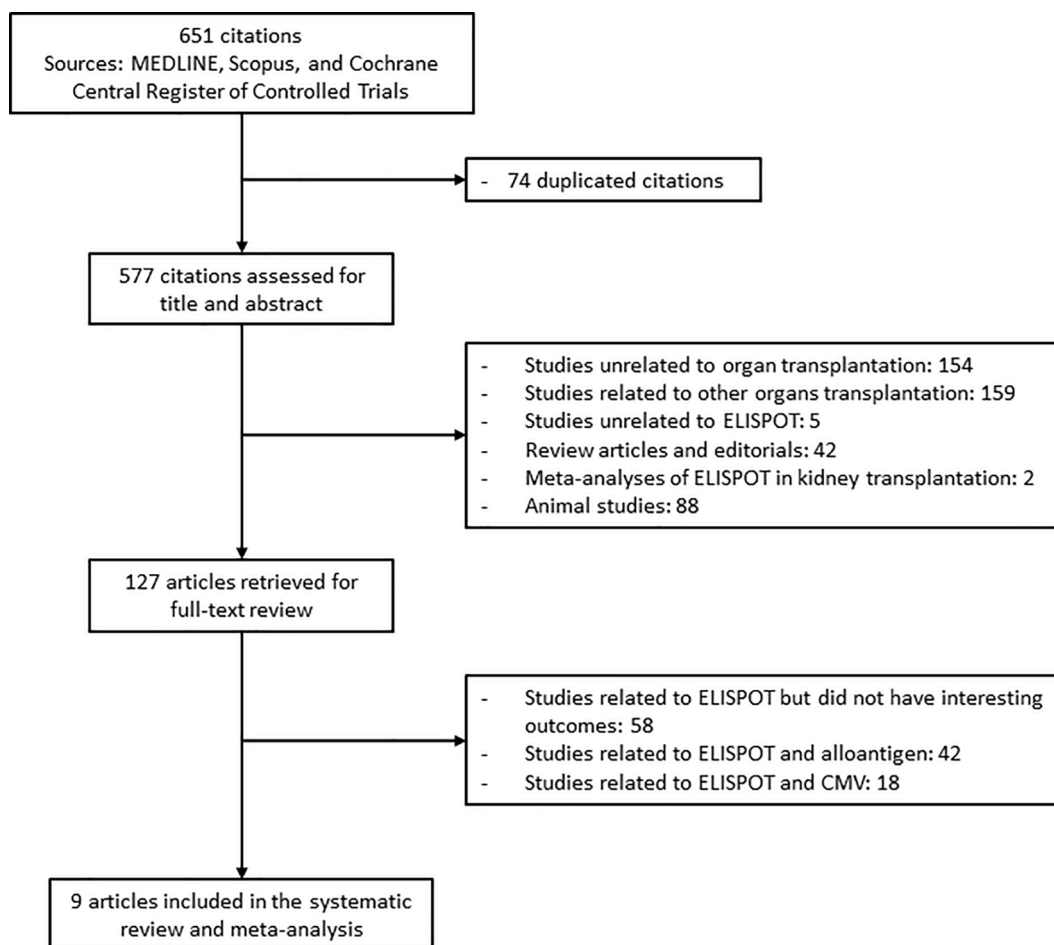Tools to monitor a patient's immune response against BKV during

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Fig. 1. Flow diagram of the study selection.

immunosuppressive therapy are lacking. Previous studies showed that interferon-γ (IFN-γ) is a cytokine with broad antiviral activities, including BKV, and that its expression is increased in response to BKV infection [16,17]. The enzyme-linked immunosorbent spot (ELISPOT) assay has been developed to evaluate T lymphocyte function (*i.e.* the frequency of IFN-γ producing responsive cells) against BKV [18-20]. Patients with BKV infection can then be stratified into risk groups. Those with the negative BKV ELISPOT, i.e. the high-risk group, who have an insufficient immune response against BKV and are therefore more likely to have persistent BK viremia and develop BKVAN [21]. On the contrary, patients with positive BKV ELISPOT have an adequate immune response against BKV, and are considered to be at low-risk for BKV-related complications [20,22].

The objective of the present study was to systematically analyze publications describing the clinical use of IFN-γ ELISPOT in kidney transplant recipients who experienced BKV replication, and provide an evidence-based assessment on whether this ELISPOT assay can be applied in clinical practice.

2. Materials and methods

2.1. Data sources and searches

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [23]. The literature search was conducted in MEDLINE, Scopus, and the Cochrane Central Register of Controlled Trials to identify eligible studies on 21 August 2020. We also manually reviewed the references listed in the retrieved articles.

For MEDLINE, we used the following Medical Subject Heading terms (MeSH): ("Enzyme-Linked Immunospot Assay"[Mesh]) AND "Kidney Transplantation"[Mesh]. For Scopus, the following search terms were applied: TITLE-ABS-KEY (ELISPOT AND Transplantation). For the Cochrane Central Register of Controlled Trials, we use the MeSH descriptor which exploded all trees of [Enzyme-Linked Immunospot Assay] and [Kidney Transplantation].

2.2. Study selection

Retrospective and prospective studies that investigated the use of ELISPOT in kidney transplantation were included. Only studies that applied the ELISPOT assay for the monitoring of immune responses against BKV were included in the meta-analysis. Studies that did not correlate ELISPOT to BKV-related clinical endpoints were not included. The ELISPOT assay could be used in both pre-transplantation and post-transplantation studies. In order to analyze the sensitivity and specificity, included studies had to report cutoff values of their ELISPOT assays. The total number of patients with and without BKV replication, and the number of patients with positive or negative ELISPOT assay results had to be reported or had to be calculated from the information provided in the manuscripts. Studies that reported the actual number of the IFN-γ producing cells in the ELISPOT assay, either at the individual patient level or reporting the mean of the study population, were also included in the analysis of the standardized mean difference (SMD). Only studies with adequate information, in accordance with the Standards for Reporting of Diagnostic Accuracy Studies (STARD) 2015[24] were included in the review. Two authors (S.U. and S.K.) independently screened the titles and abstracts of the electronic citations, and full-text

Table 1

Summary of studies reporting data of IFN-γ ELISPOT assays in BKV-infected kidney transplant recipients.

Reference	Authors and year of publication	Country of origin	Timing of ELISPOT	Cutoff value after subtraction of negative control	BK antigen used for ELISPOT assay	Patient with active BKV infection (n)	Patients with resolving BKV infection (n)	Onset of BK viremia (months after transplantation)	Patients who never had BKV infection (n)	Definition of BKV infection
[27]	Binggeli et al. 2007	Switzerland	Post-transplantation	69 IFN-γ pc per 10^6 PBMC	Large T, VP1	22	20	N/A	–	Viremia
[28]	Prosser et al. 2008	USA	Post-transplantation	not mentioned (IFN-γ pc per 10^4 PBMC)	Large T	8	8	16 ± 11	–	BKV-associate nephropathy
[29]	Chakera et al. 2011	UK	Post-transplantation	50 IFN-γ pc per 10^6 PBMC	Large T, small t, VP1, VP2, VP3	9	9	N/A	8	Urine decoy cell and viremia
[30]	Schachtner et al. 2011	Germany	Post-transplantation	10 IFN-γ pc per 10^6 PBMC	Large T, small t, VP1, VP2, VP3	18	17	14 ± 18	–	Viremia
[31]	Costa et al. 2014	Italy	Post-transplantation	5 IFN-γ pc per 2×10^5 PBMC	Mixed antigen	12	–	N/A	137	Viruria or viremia
[32]	Schachtner et al. 2014	Germany	Post-transplantation	10 IFN-γ pc per 2.5×10^5 PBMC	Large T, small t, VP1, VP2, VP3	12	12	2 ± 1	17	Viruria or viremia
[33]	Mutlu et al. 2015	Turkey	Pre- and post-transplantation	10 IFN-γ pc per 2×10^5 PBMC	Mixed antigen	12	6	5 ± 2	26	Viremia
[34]	Schachtner et al. 2015	Germany	Pre- and post-transplantation	25 IFN-γ pc per 3×10^5 PBMC	Mixed antigen, large T, VP1	16	–	3 ± 4	92	Viremia
[35]	Bae et al. 2020	South Korea	Post-transplantation	not mentioned (IFN-γ pc per 3×10^5 PBMC)	Large T, small t, VP1, VP2, VP3	17	34	13 ± 14	17	Viremia

n: number.

pc: producing cells.

N/A: not available.

articles were retrieved for comprehensive review, and were independently rescreened. Disagreements were resolved through consensus and arbitration by D.H. and C.B.

2.3. Data extraction and quality assessment

The following data were extracted from each study: author's name, year of publication, country of origin, type of study, timing of ELISPOT testing, the total number of patients with positive and negative ELISPOT tests, and the number of patients with BKV replication in each group. The cutoff for a positive ELISPOT assay in each study and the patients' actual number of IFN-γ producing cells were included for the analyses. In the post-transplantation ELISPOT studies, the ELISPOT results were retrieved from 2 time points. The first measurements were at the time that BKV replication was diagnosed or when viral load was actively increasing, which in our review we defined as having the "active BKV infection". The second ELISPOT results were collected when the infection was resolving or closest to the time of BK viral clearance, which we defined as "resolving BKV infection". If the ELISPOT values at these time points were not available, the actual values in these studies were not included in the analyses. Every type of BKV antigen used for stimulating the recipient's peripheral blood mononuclear cells (PBMC) was covered, including large T, small t, virion protein 1 (VP1), VP2, VP3, and mixed BK antigen. Patient characteristics, including immunosuppressive regimen and allograft function, were obtained from each study if available.

The Quality Assessment of Diagnostic Accuracy Studies tool (QUADAS-2)[25] was used to evaluate the risk of bias. This tool contains 4 domains, which are patient selection, index test, reference standard, and flow and timing.

2.4. Data synthesis and analysis

As global measures of accuracy across all test threshold, we calculated the pooled diagnostic odds ratio (OR) for active BKV infection in patients with positive IFN-γ ELISPOT compared with patients who had negative IFN-γ ELISPOT, and the area under the summary receiver operating characteristic (SROC) curve. In calculating the OR, a continuity correction was applied to all cells in studies with any zero-cell count. The standardized mean difference (SMD) of the IFN-γ producing cells from patients with active and resolving BKV infection were calculated after normalizing the actual value of the ELISPOT assay to the number of IFN-γ producing cells per 3×10^5 PBMC. For studies not providing mean and standard deviation (SD), the estimation method by Wan et al. was applied [26]. All pooled estimates were calculated using random effects models. A funnel plot was used to demonstrate possible publication bias, and Egger's method was used to test for asymmetry of the funnel plot. The existence of heterogeneity among study effect sizes was examined using the I^2 index and the Q-test p-value. An I^2 index higher than 75% reflects medium to high heterogeneity. The analyses were performed using Stata Statistical Software Release 15.1 with the user written commands midas, metandi and metadta (StataCorp LLC,

Table 2
QUADS-2 for risk of bias assessment.

Authors and year of publication	Risk of bias				Applicability concerns		
	Patient selection	Index test	Reference standard	Flow and timing	Patient selection	Index test	Reference standard
Binggeli et al. 2007	Low	High	Low	Low	Low	Low	Low
Prosser et al. 2008	Unclear	Low	Low	Low	Low	Low	Low
Chakera et al. 2011	High	Low	Low	Low	Low	Low	Low
Schachtner et al. 2011	Low	Low	Low	Low	Low	Low	Low
Costa et al. 2014	High	Low	Low	High	High	Low	Low
Schachtner et al. 2014	Low	Low	Low	Low	High	Low	Low
Mutlu et al. 2015	High	Low	Low	High	High	Low	Low
Schachtner et al. 2015	Low	Low	Low	Low	Low	Low	Low
Bae et al. 2020	Low	Low	Low	Low	Low	Low	Low

College Station, TX) and GraphPad Prism version 9.0.0 for Windows (GraphPad Software, San Diego, CA).

2.5. Ethical considerations

This meta-analysis and systematic review did not directly obtain data from human or animal subjects. All of the included studies' information was published in the scientific journals without the possibility to identify the individual patients.

3. Results

3.1. Characteristics of the studies

A total of 651 articles were identified. The flow diagram of the included and excluded studies is shown in Fig. 1. After exclusion of irrelevant and duplicated studies, 127 articles underwent full-text review. Nine articles fulfilled the inclusion criteria and were included in the meta-analysis [27-35]. The summary of study characteristics is
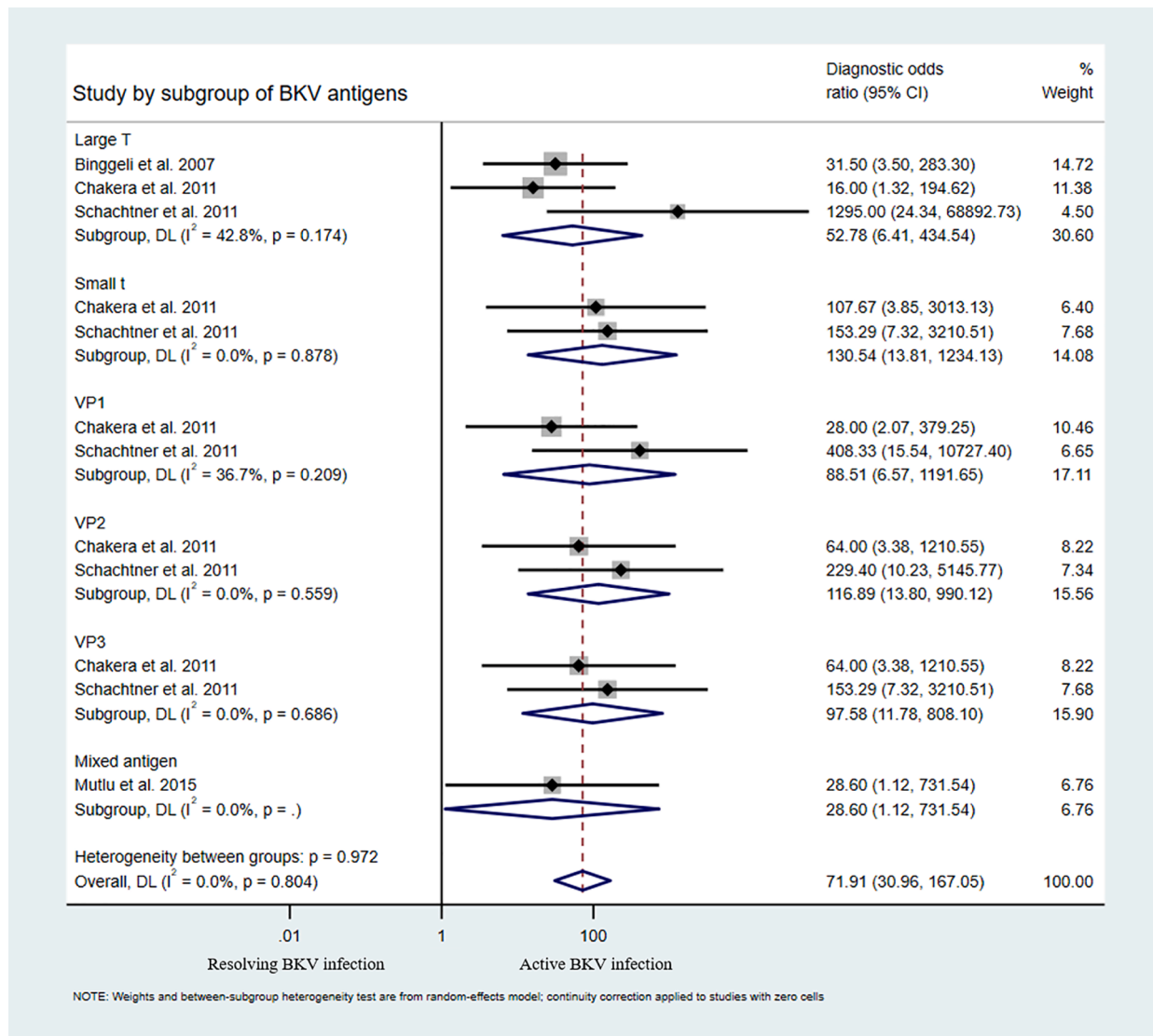

Fig. 2. Diagnostic odds ratio of patients with negative compared to positive BKV-specific IFN-γ for having active BKV infection.
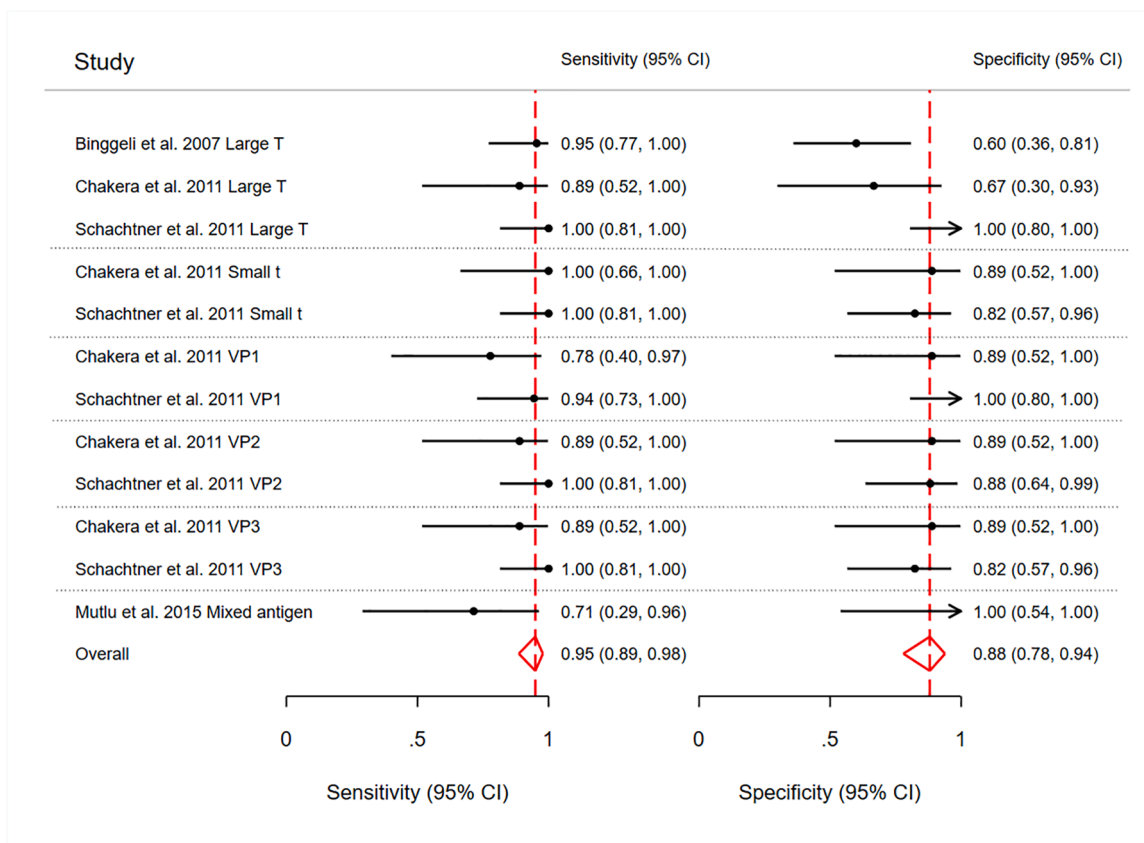
Fig. 3. Sensitivity and specificity of the BKV-specific IFN-γ ELISPOT assay for the diagnosis of active BKV infection.

shown in Table 1. All studies evaluated the relationship between the IFN-γ ELISPOT assay and BKV infection in the post-transplantation period. Two of these nine studies also assessed pre-transplantation measurements [33,34]. The nine studies varied in study design and technical approach. First, the BK viral antigens used for each study varied, including large T antigen in 7 studies, small t antigen in 4 studies,

VP1 antigen in 6 studies, VP2 antigen in 4 studies, VP3 antigen in 4 studies, and mixed antigen in 3 studies. All studies measured IFN-γ as the cytokine for the T lymphocyte-specific immune response against BK viral antigens. Second, the definitions of BKV infection among these studies were different, and ranged from positive decoy cells in urine, viruria, viremia, to full-blown BKVAN. Third, the number of PBMCs used
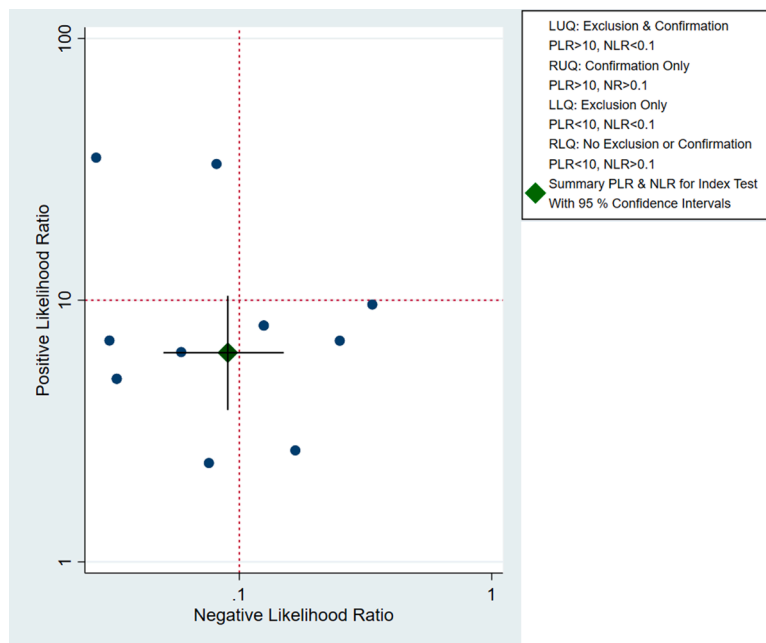

Fig. 4. Scatter plot of the positive likelihood ratio (PLR) and the negative likelihood ratio (NLR) of having active BKV infection, when patients with negative BKV-specific IFN-γ ELISPOT were considered at risk of active BKV infection.
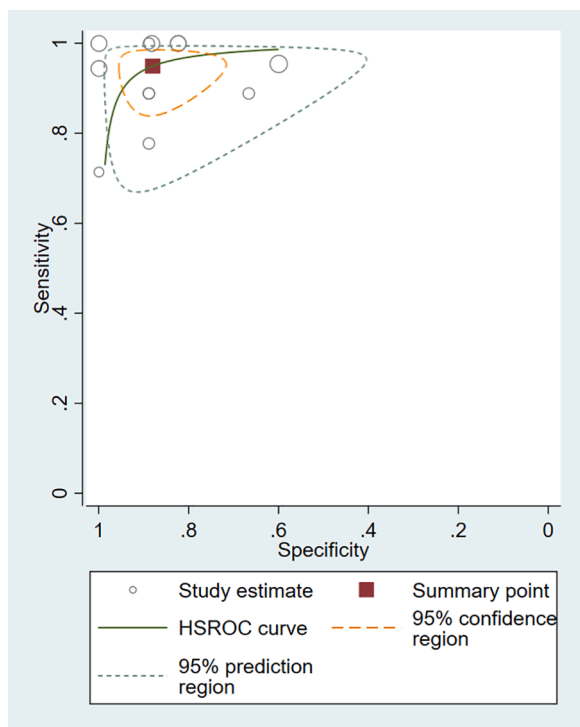
Fig. 5. The hierarchical summary receiver operating characteristic (HSROC) curve of BKV-specific IFN-γ ELISPOT assay against BK viral antigens.

in the IFN-γ ELISPOT assay varied substantially. To compare the outcomes of the different studies, we normalized the actual value of the ELISPOT assay to the number of IFN-γ producing cells per 3×10^5 PBMC. The QUADS-2 risk of bias assessment for each study is shown in Table 2.

Considering that only 2 studies[33,34] evaluated the BKV ELISPOT assay before kidney transplantation, we decided to focus our analyses on publications reporting post-transplantation studies. The included studies used varying definitions of BKV infection as shown in Table 1, including decoy cell-positive, viruria, viremia, or BKVAN. Each study measured IFN-γ ELISPOT response to BK viral antigen and correlated this with the clinical course of the patients, which was classified as "active BKV infection" and "resolving BKV infection". Four studies[27, 29,30,33] reported sufficient information on the ELISPOT cutoff values, the number of patients with positive and negative ELISPOT test results, and the BKV infection status at the time that ELISPOT was tested, which allowed us to perform meta-analysis for the diagnostic efficacy. The pooled ELISPOT values in patients with active BKV infection compared with patients who had resolving BKV infection was formulated from 7 studies[27-30,32,33,35], which could also be used for the calculation of the SMD. One study[31] did not report adequate information on the timing of the ELISPOT assay and patients' BKV status, and therefore was excluded from the final analysis.

3.2. Post-transplantation ELISPOT assay for the monitoring of BKV infection

We examined the patients who had BKV infection, including patients with active BKV or resolving BKV infection, and compared the IFN-γ ELISPOT results between these groups. Patients who had IFN-γ ELISPOT values less than the cutoff were regarded as having a "negative ELISPOT", whereas patients with an IFN-γ ELISPOT test result higher than the cutoff were considered as having a "positive ELISPOT". The cutoff values used in each study were different as shown in Table 1. Patients with negative ELISPOT were at higher risk for active BKV infection compared to patients with positive ELISPOT who were more likely to

have resolving BKV infection (diagnostic OR 71.91, 95%-CI 30.96–167.05, p-value < 0.001, $I^2 = 0\%$, Q-test p-value = 0.80; Fig. 2). The results were consistent in every subgroup of BK viral antigen studied. Fig. 3 displays the sensitivity and specificity of the ELISPOT assay for detecting patients with active BKV infection. Overall, the ELISPOT assay performed with pooled sensitivity of 0.95 (95%-CI 0.89–0.98) and specificity of 0.88 (95%-CI 0.78–0.94). When a negative ELISPOT was considered to indicate a positive result for the risk of active BKV infection, the pooled positive likelihood ratio (PLR) was 6.3 (95%-CI 3.8–10.4) and the pooled negative likelihood ratio (NLR) was 0.09 (95%-CI 0.05–0.15; Fig. 4). The hierarchical summary receiver operating characteristic (HSROC) curve was analyzed (Fig. 5), which had an area under the HSROC curve of 0.97 (95%-CI 0.95–0.98). Finally, the funnel plot of the log of OR was not completely symmetrical, although the observed studies were within the 95%-CI (Supplementary Figure S1). Egger's test also suggested there was evidence of plot asymmetry with p-value of 0.01.

3.3. Differences in IFN-γ ELISPOT test results between patients with active and resolving BKV infection

We calculated the SMD of the IFN-γ ELISPOT value between the patients with active BKV infection as compared with patients who had resolving BK infection. Fig. 6 summarizes the SMD from each study and shows the pooled SMD of −2.09 (95%-CI −2.50, −1.68, p-value < 0.001, $I^2 = 79.4\%$, Q-test p-value < 0.001). To illustrate the difference between patients with active and resolving BKV infection, mean and SD of the number of IFN-γ producing cells (pc) was plotted for each BK viral antigen used. Supplementary Figure S2 shows mean ± SD of the number of IFN-γ pc from the large T and small t antigen, in the patients with active BKV infection compared with the patients with resolving BKV infection (8.3 ± 7.8 vs. 34.4 ± 25.7 IFN-γ pc/3×10^5 PBMC, and 6.6 ± 8.6 vs. 34.9 ± 20.7 IFN-γ pc/3×10^5 PBMC, respectively). The number of IFN-γ pc that responded to VP1, VP2, and VP3 antigen are described in Supplementary Figure S3 (18.2 ± 22.6 vs. 56.8 ± 56.0 IFN-γ pc/3×10^5 PBMC, 7.1 ± 7.7 vs. 33.2 ± 16.5 IFN-γ pc/3×10^5 PBMC, and 7.0 ± 8.1 vs. 36.0 ± 23.3 IFN-γ pc/3×10^5 PBMC, respectively).

4. Discussion

This study is the first systematic review with meta-analysis of the IFN-γ ELISPOT assay that correlated T cell BKV responsiveness with clinical BKV infection in kidney transplant recipients. Overall, the IFN-γ ELISPOT assay has a high capability to discriminate patients with active BKV infection from patients with resolving BKV infection. Patients with negative IFN-γ ELISPOT were at a 71.9-fold higher risk to have active BKV infection compared to the patients with positive IFN-γ ELISPOT. However, the test has NLR of 0.09 and PLR of 6.3, which signifies that the assay is suitable for exclusion, but not for confirmation of active BKV infection.

BKV is an important causes of kidney allograft loss due to the lack of effective treatment [21,36,37]. The current practice is to prevent significant BKV replication, including the surveillance for early BK viremia or viruria and minimizing risk factors that are known for BKV reactivation [10,15,38]. Besides the well-recognized risks such as intensified immunosuppression, the degree of human leukocyte antigen (HLA) mismatches, and kidney allograft ischemic reperfusion injury [7,10,12, 13,36], more recent evidence suggests that the mismatch between high donor BKV IgG sero-reactivity and low recipient sero-reactivity significantly increases the risk of BK viremia [39]. Moreover, the lack of donor-specific BKV genotype neutralizing antibody in the recipient also significantly associated with BKV infection after transplantation [40]. To control BKV replication, cells of both innate and adaptive immunity are involved. BKV-specific CD4+ T lymphocytes suppress BKV by the production and secretion of proinflammatory cytokines, particularly IFN-γ, tumor necrosis factor (TNF), and the serine protease granzyme B
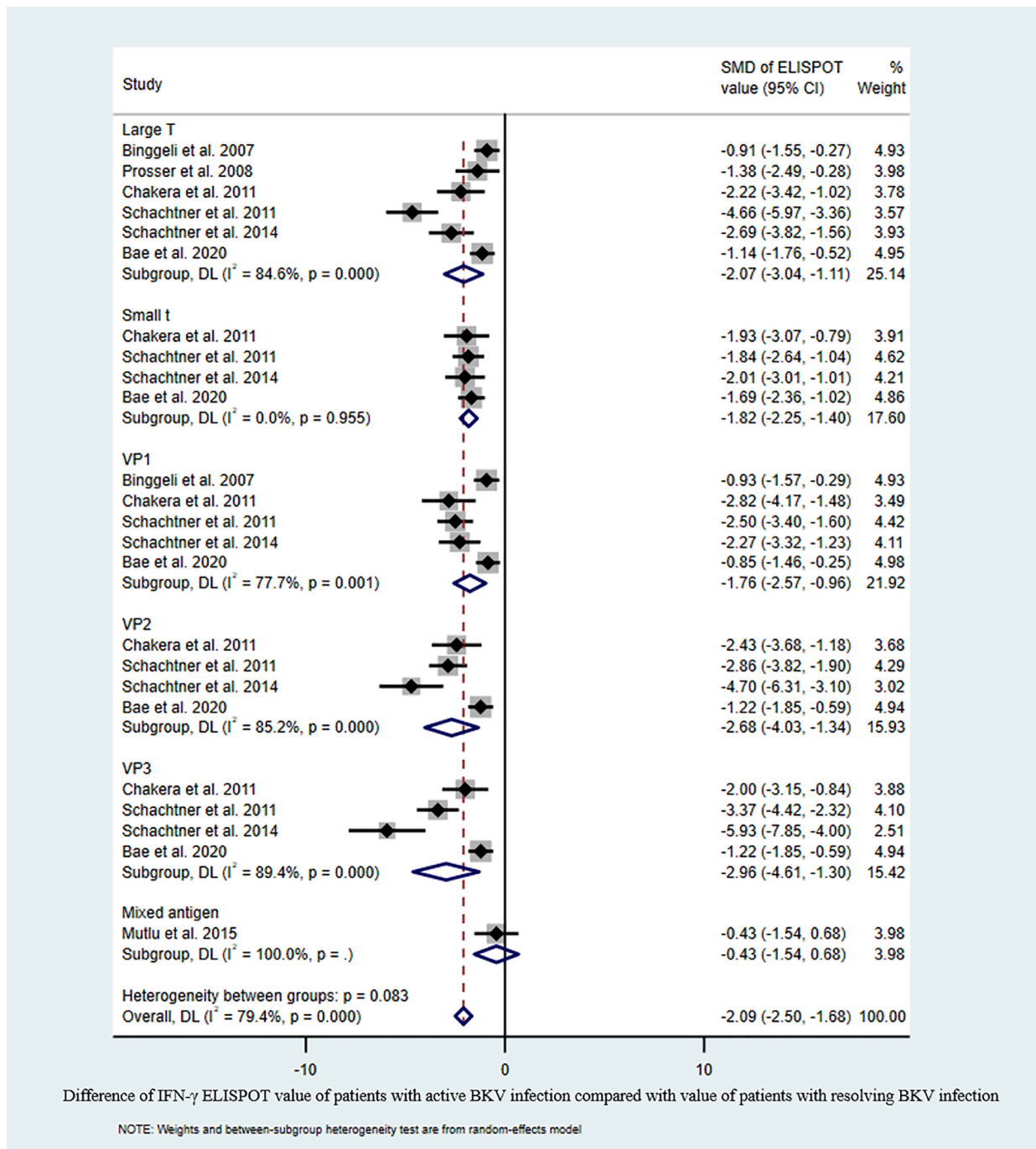
Fig. 6. The standardized mean difference (SMD) of the IFN-γ ELISPOT value between patients with active BKV infection and patients with resolving BKV infection.

[41]. Different BKV antigens also activate different subsets of T lymphocytes, for example; VP1 mainly activated CD4+ *T* lymphocytes, while large T antigen stimulates CD8+ *T* lymphocytes [27]. In our view, the results of the BKV-specific ELISPOT could assist the clinician in monitoring and treating of BKV by two approaches.

First, by performing an anti-BKV IFN-γ ELISPOT assay at the time that BK viruria or viremia is first detected. By this method one will be able to classify patients as having a positive or negative ELISPOT result. The former will be more likely to have self-limited BKV replication, transient BK viremia, and a good prognosis without substantial changes to their immunosuppressive treatment. On the contrary, patients with negative ELISPOT will possibly progress to persistent BK viremia or BKVAN and may therefore need more aggressive interventions [21].

Second, another suitable period is when immunosuppressive reduction has been implemented for the treatment of BKV infection. The IFN-γ ELISPOT assay could then serve as to guide the clinician whether or not to lower the immunosuppressive medications. Patients who have increasing numbers of IFN-γ pc to BKV continuously since the beginning of the intervention are more likely to eventually clear the virus. The number of BKV-specific IFN-γ pc that a clinician should target are depicted in Supplementary Figures S2 and S3 which should be accompanied by the patients' clinical course.

This ELISPOT technique is a highly sensitive method for the quantification of cytokine-producing cells after stimulation with a stimulus which in this case is a BKV antigen [42]. All included studies standardized their ELISPOT assay by including positive controls, either the

superantigen *Staphylococcus enterotoxin* B or phytohemagglutinin, and negative controls. A few studies reported that the BKV antigens can cross-react with JC virus [43-45] which is an extremely rare cause of allograft nephropathy [46]. Other limitations of the ELISPOT assay are that it is time-consuming, relatively expensive, and that there is a need for well-trained personnel. Also, this assay does not provide information about the cellular source of the BKV-specific response. For this, flow cytometry is the better technique. Using intracellular cytokine staining by flow cytometry, Ahlenstiel-Grunow T. et al. showed that the high amount of CD4+ and CD8+ BKV-specific T lymphocytes are of importance to control the virus and prevent BKVAN [47]. Moreover, the poly-functional CD8+ *T* lymphocytes which secrete IFN-γ, interleukin 2 (IL-2), and TNF-α are needed for BKV clearance, while the mono-functional CD8+ *T* lymphocytes are only effective for suppression of low-level BK viremia [48]. Altogether, the information from the ELISPOT assay and the amount of BKV-specific T lymphocytes and their functions, should be included in future trials exploring the value of these techniques in management strategy of BKV infection.

BKV-specific IFN-γ ELISPOT should be interpreted together with the BK viral load results. In current clinical practice, it is difficult to differentiate BK viremic patients who will achieve BK viral clearance from those who will have progressive BK viremia leading to BKVAN. Knowing the ELISPOT result at the time of viral load testing, would allow clinicians to make informed decisions based on the patient's immune response against BKV. Patients could then be stratified as high or low risk for developing BKVAN, and immunosuppression adjusted accordingly. This add-on value of the ELISPOT to supplement BK viral load testing would help prevent unnecessary aggressive immunosuppression reduction that leads to concurrent or superimposed acute rejection in patients with BKV infection, a scenario which remains problematic in kidney transplantation. However, since there are variations of BKV-specific IFN-γ ELISPOT protocol among laboratories, including the type of BKV antigens used, the amount of recipient's PBMC used in the assay, and the cutoff values. Development of a standardized protocol with evidence-based threshold cutoffs to defined antigens and consistent PBMC concentrations is still needed before this method can be routinely applied in the clinics.

Our study is not without limitations. The funnel plot and Egger's p-value indicate that there may be publication bias in the meta-analysis. Further studies are needed to confirm or reject our findings. Second, the studies of pre-transplantation number of BKV-specific IFN-γ pc were too few to include in the meta-analysis. Interestingly, Schachtner et al. showed that the risk of BKV reactivation could be predicted by the loss of pre-transplant BKV-specific immunity in the post-transplantation period [34]. These findings were in contrast with the results from Mutlu et al. which did not find an association between pre-transplantation BKV-specific immunity and the development of post-transplantation BKV reactivation [33]. Further studies are required regarding the evaluation of pre-transplantation BKV-specific immunity and choosing a particular immunosuppressive regimen. Third, we realize that the non-normal distribution of the number of BKV-specific IFN-γ pc might have caused bias in the estimation of the mean and SD. Nevertheless, we have used methods that provide the best estimate of the sample mean and SD from the skewed data [26]. In addition, to minimize to risk of bias from outlier values, the cutoff of the ELISPOT assay was used to classify patients as having positive and negative results, and was correlated with the clinical evidence of BKV reactivation.

In conclusion, this meta-analysis and systematic review demonstrates that the IFN-γ ELISPOT assay is a useful tool for assessing the post-kidney transplantation risk of BKV-associated complications. Patients with an adequate T lymphocyte BKV-specific immune response (positive ELISPOT) are more likely to achieve resolution of BKV infection.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2021.104848.

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